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The major cause of cancer deaths can be attributed to metastasis. Our goal was to identify metastasis-controlling genes for human breast cancer. This research is based upon our finding that microcell-mediated transfer of chromosome 11 into MDA-MB-435 results in nearly complete suppression of metastasis without suppressing tumorigenicity.

The key findings during this reporting period were: (1) identification of three novel genes which are more highly expressed in metastasis-suppressed human breast carcinoma cell lines; (2) demonstration that one of the novel genes, designated BrMS1 (Breast Metastasis Suppressor-1), suppresses breast cancer metastasis in a dose-dependent manner; (3) BrMS1 maps to 11q13, a site commonly altered in late-stage breast carcinoma; (4) Preliminary results include: (1) Full-length for one of the two other novel cDNAs has been cloned. Transfections have been initiated and colonies are beginning to appear. Despite several attempts, we have been unsuccessful in obtaining full-length for the third novel gene.

In short, we have essentially completed the major objective of this proposal and are completing initial characterization of the candidate metastasis suppressor gene.

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FOREWORD

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Danny R. Welch — 5 July 99

PI - Signature — Date

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INTRODUCTION

Experimental Context – The most dangerous attribute of cancer cells is their ability to spread (metastasize). Our objective is to determine the molecular mechanisms responsible for controlling breast cancer spread. The timing and location of nonrandom karyotypic abnormalities has provided clues regarding the genes involved in breast carcinoma progression. In breast cancer, structural changes frequently involve chromosomes 1, 6, 8, 11, 13, 16 and 17. Chromosomes 8, 13 and 17 changes generally occur early in progression; whereas, deletions and rearrangements of chromosomes 1, 6, 11 and 16 often occur later (reviewed in (Welch and Wei, 1998)). As a corollary, one would hypothesize that genes relevant to breast cancer progression toward metastasis are encoded on the latter chromosomes. To test this hypothesis, we introduced an intact, normal human chromosome 11 into the metastatic human breast carcinoma cell line, MDA-MB-435 using microcell-mediated chromosome transfer (MMCT). We showed that metastasis was suppressed by 95%, but tumorigenicity was unaffected (Phillips *et al.*, 1996). This finding suggested the presence of at least one human breast carcinoma metastasis-suppressor gene on chromosome 11. *Please note*: We define a metastasis-suppressor gene as blocking tumor spread without suppressing tumorigenicity. A *tumor* suppressor gene would suppress tumor growth and, by inference, metastasis as well.

The goals of DAMD-17-1-96-6152 were to map and/or clone a gene(s) on chromosome 11 responsible for metastasis suppression. In addition, we wanted to test whether similar metastasis suppression occurs if chromosome 11 is introduced into other metastatic human breast carcinoma cell lines. These technical objectives fall within the ultimate goal of understanding the mechanisms underlying breast cancer metastasis.

Background – Metastasis results from accumulated genetic changes from which a subset of late-stage cancer cells evolve that are no longer confined to their tissue of origin for growth. In order to successfully colonize a distant organ, metastatic cells must survive transport through the body, interact with a variety of host cells and successfully penetrate numerous barriers. If a cell cannot complete every step, it is nonmetastatic. The multistep metastatic cascade involves numerous genes (Ahmad and Hart, 1997;Beckmann *et al.*, 1997;Price *et al.*, 1997;Welch and Goldberg, 1997;Welch and Wei, 1998). Two classes of metastasis-associated genes have been identified — (i) genes that drive metastasis formation, and (ii) genes that inhibit metastasis. However, the identities of most of these genes remain unknown. Correspondingly, it is not known how these genes are regulated in normal and/or cancer cells. Nonetheless, it is well recognized that the probability for long-term survival is extremely low if metastases develop.

In addition to the findings mentioned above, we have made four observations relevant to the genetics of human breast cancer metastasis. (1) The melanoma metastasis-suppressor gene KiSS1 also suppresses metastasis of human MDA-MB-435 cells. (2) The prostate carcinoma metastasis-suppressor gene Kai1 also suppresses metastasis of human MDA-MB-435 cells. (3) Introduction of a normal human chromosome 11 into human MDA-MB-435 cells suppresses metastasis by at least 90%.

BODY

Positional cloning has been used to identify a number of tumor-suppressor genes (e.g., WT1, Rb, FHIT) and genes for mutations that predispose cancer susceptibility (e.g, NF1, APC) (reviewed in (Stanbridge, 1990)). As mapping nears completion, detection of mutations among cancer families confirms a particular gene's role as a tumor suppressor. Since mutations are relatively rare, equally strong evidence for a role in cancer etiology is required. Thus, positional cloning is reasonable if strong, well-characterized pedigrees are available. However, determining roles for genes in sporadic tumors or progression-associated genes (e.g., metastasis-controlling) is difficult because of tumor heterogeneity,

genetic instability and the huge number of experiments necessary to prove causality. This is further complicated for multigenic, complex phenotypes, like metastasis. Simply, the statistical likelihood for identifying a specific gene over the immense background of genetic instability typical of late-stage tumors is difficult. Thus, alternative approaches are required.

MMCT (microcell-mediated chromosome transfer) has provided functional evidence for tumor suppressor genes when other approaches have failed (Anderson and Stanbridge, 1993;Hunt, 1996). The functional data have provided the necessary information for successful mapping of the genes responsible (Saxon *et al.*, 1986;Trent *et al.*, 1990;Loh *et al.*, 1992;Church *et al.*, 1993;Chen *et al.*, 1994;Coleman *et al.*, 1995;Ewing *et al.*, 1995;Ohmura *et al.*, 1995;Theile *et al.*, 1995;England *et al.*, 1996;Casey *et al.*, 1997;Gioeli *et al.*, 1997;Koi *et al.*, 1997;Kuramochi *et al.*, 1997;Reiss *et al.*, 1997;Kon *et al.*, 1998;Robertson *et al.*, 1998). As an intermediate, some have utilized a modification of MMCT in which the donor chromosome has been irradiated to produce deletions (Dowdy *et al.*, 1990;Koi *et al.*, 1993;Coleman *et al.*, 1995;Murakami *et al.*, 1995;Gioeli *et al.*, 1997;Plummer *et al.*, 1997). This modification is based upon a loss of function (i.e., failure to suppress) associated with the deletion.

The strategies we proposed for identifying metastasis-controlling genes in human breast cancer were based upon those listed above as well as those we used to identify novel metastasis-suppressor genes in human melanoma (Welch *et al.*, 1994;Jiang *et al.*, 1995;Lee *et al.*, 1996;Lee and Welch, 1997;Miele *et al.*, 1997). Basically, two concurrent approaches were outlined. First, progressively smaller fragments of neo-tagged human chromosome 11 were to be introduced into MDA-MB-435 by MMCT. By evaluating regions of overlap for chromosomal fragments present/absent in suppressed/non-suppressed hybrids, the location of the putative metastasis-suppressor gene(s) would be defined. The second approach was to use differential display (Liang and Pardee, 1992;Liang *et al.*, 1993)and subtractive hybridization (Ausubel *et al.*, 1990;Hutchins *et al.*, 1991). Once candidate genes were identified, transfections and testing for metastasis in appropriate animal models would confirm that a *bona fide* metastasis-suppressor gene had been cloned.

The second major objective of DAMD-17-1-96-6152 was to demonstrate the introduction of chromosome 11 into another metastatic human breast carcinoma also suppresses metastasis.

This progress report will be organized in sections. Each section summarizes results from related series of experiments and the relationship of those experiments to a particular Specific Aim is noted. Only new data, collected since submission of the FY97-98 progress report, is included.

Section 1: Welch, D.R. and Wei, L.L. (1998) Molecular control of breast cancer progression and metastasis. *Endocrine Related Cancers* **5**: 155-196.

Summary: This was an invited paper in which I was asked to review briefly the literature about metastasis-controlling genes in human breast cancer, particularly genes that are hormonally regulated. However, there were over 8000 papers in the literature which claimed to present data showing association between metastasis and particular genes. This necessitated that the breadth of the review be expanded in order to review the role of genes in breast cancer at "all" stages of progression. Basically, most papers speculated a role of genes in invasion, progression and metastasis but presented no data to support such claims. Additionally, the problems associated with ill-defined model systems (i.e., what kind of breast cancer is being studied?) was addressed.

While not directly addressing a specific aim from the original proposal, this review was extremely useful for formulating and modifying my thinking about breast cancer genetics. During the writing process, I had to address many issues related to breast cancer metastasis research and organize them. The critical review also helped us focus on key issues which need to be addressed in order to accomplish the

aims set forth for this program.

Section 2: MMCT of pieces of chromosome 11 into MDA-MB-435 [Unpublished]

This results reported in this section are based upon the strategy proposed for Specific Aim 1 in the original proposal. The goal of this aim is to introduce progressively smaller pieces of chromosome 11 in order to map gene(s) responsible for metastasis suppression.

Our initial approach was to prepare chromosome 11 microcell donors that have deletions as a result of radiation damage (Dowdy *et al.*, 1990;Bader *et al.*, 1991;Robertson *et al.*, 1996;Robertson *et al.*, 1997). Deletion mutants would then be introduced by MMCT into MDA-MB-435 followed by assessment of metastasis in athymic mice. With this approach, random deletions need not be mapped beforehand. They could be mapped following fusion based upon predetermined polymorphisms spanning chromosome 11. If the metastasis-suppressor gene is retained, functional complementation of the defect would be repaired and the cells would be nonmetastatic. If the gene had been deleted, suppression would not occur. Metastatic hybrids would then be evaluated for portions of the chromosome 11 retained. Position of the metastasis-suppressor gene could be inferred by the smallest region of shared deletion. This has most recently been used to clone tumor or growth suppressor genes for a variety of cancers (Murakami *et al.*, 1995;Kawana *et al.*, 1997;Plummer *et al.*, 1997;Reiss *et al.*, 1997;Chekmareva *et al.*, 1998;Mashimo *et al.*, 1998;Uejima *et al.*, 1998;Uzawa *et al.*, 1998;Robertson *et al.*, 1999;Gao *et al.*, 1999;Nihei *et al.*, 1999). We recently used this approach and verified that the PTEN/MMAC1 phosphatase gene functions as a tumor suppressor in some human melanoma cell lines (Robertson *et al.*, 1998).

The second approach is to utilize MMCT donors with previously defined fragments of chromosome 11 (Chen *et al.*, 1995;Robertson *et al.*, 1996;Robertson *et al.*, 1997). The advantage of this approach is that fully-defined DNA is introduced into the cells. While aesthetically pleasing, the time required to fully characterize the donor chromosome fragment can take months to years.

Initially, the second approach was only to be a contingency because characterization of chromosome donors is highly labor intensive. However, we have a collaboration with Dr. Jane Fountain (University of Southern California) where our objective is to map melanoma tumor suppressor genes on chromosome 11 (Robertson *et al.*, 1997). Unfortunately, similar problems have been encountered for the melanoma transfer experiments that have been encountered for the breast MMCT.

After several discussions, Dr. Fountain and I have concluded that it will be worthwhile to take advantage of the large body of published positional cloning (loss of heterozygosity) data from clinical samples to map hot spots in breast cancer. This would identify large-insert vector forms (P1, PAC, BAC, YAC ...) which could then be retrofitted with selectable markers. Vectors are now available to retrofit P1, BAC or PAC clones 11(Mejia and Monaco, 1997). Based upon their relatively large average insert size of BAC/PAC/P1 (100-200 kb), it has become feasible to individually transfect P1 or PAC clones into breast carcinoma cells. Even with 3 chromosomal regions of 1 Mb each (total 3 Mb), the maximum number of transfectants would be 300. Although this number is not trivial, we estimate that the quantity of work for retrofitting and transfection would be estimated in months rather than years for the chromosome pieces. Given that the efficiency of transformation with these vectors is more efficient than MMCT, the probability for success would be higher. In addition, the P1, BAC and PAC clones have relatively low recombination frequencies (unlike YAC and even chromosome fragments), making their use "safer" for introduction into mammalian cells. Since the chromosome pieces are generated using radiation, we always run the risk of false negative results because an (in)active point mutant has been introduced.

We did obtain a retrofit vector, but our analysis did not correspond to the published sequence. After

losing approximately four months, a replacement was obtained. We have now successfully introduced a BAC clone into this vector and have selected single cell colonies for analysis. Now that we have obtained the necessary experience for this technique, our progress should become more rapid.

Section 3: Introduction of chromosome 11 into MDA-MB-231 [Unpublished]

This objective corresponded to specific aim 2 in the original proposal. The purpose was to determine whether chromosome 11 with suppress metastasis in independently derived human breast carcinoma cell line. Since MDA-MB-231 is the only other human breast carcinoma cell line that metastasize is a high frequency, we proposed to insert chromosome 11 into MDA-MB-231.

In previous progress reports, we reported our difficulty in obtaining metastatic variants other than MDA-MB-435. The original grant proposal proposed use MDA-MB-231; however, another variants we obtained were indeed metastatic. This deficiency was rectify in summer 1998 when he received a variant of the MDA-MB-231 from Dr. Janet Price (U.T.-M.D. Anderson Cancer Center, Houston, TX). The proposed strategy, to introduce chromosome 11 into MDA-MB-231, was considered lower priority because the MDA-MB-231 exhibited no defects for chromosome 11. Several considerations contributed to this decision. First, we were experiencing difficulty in obtaining reliable chromosome 11 fragment donors. Second, we were having some success in the differential display approach, providing the opportunity to directly test individual genes in this cell line. Were we not to have success following transfection, microcell-mediated introduction of chromosome 11 into MDA-MB-231 would have been initiated.

Toward the objective of testing for metastasis suppressors in human breast cancer, we have continued to search for metastatic cell lines using three parallel approaches. (1) On an *ad hoc* basis, we have assayed *in vivo* metastatic potential of a series of breast tumor cell lines developed by Dr. Steven Ethier (University of Michigan). While two of the cell lines to develop the single regional lymph node metastasis, none were reproducibly metastatic in sufficiently high frequency to justify further study.

Since MDA-MB-435 is metastatic and heterogeneous, we decided that alternative strategies may be useful. (2) Isolation of single cell clones did not yield reproducibly more highly metastatic variants than the parental cells. (3) Likewise, despite selection for increased lung colonization potential (in a manner analogous to Fidler), no significantly more highly metastatic variant was obtained (Since the data has not substantially changed from last year's progress report, it is not included herein.). The latter two approaches have been moved to the back burner in lieu of other aspects of this research which were being more productive (see below).

Section 4: *BRMS1*, a suppressor of metastasis in human breast carcinoma (1999) M.J. Seraj, R.S. Samant, T.O. Leonard, M.F. Verderame, D.R. Welch (Submitted, *Nature Medicine*)

Results reported in this section correspond to Specific Aims 3 and 4 in the original proposal. Briefly, our approach was to use differential display in order to identify candidates which were significantly more expressed metastasis-suppressed neo11/435 hybrid cell clones (Aim 3) and test them *in vivo* for metastatic potential (Aim 4). Six candidate genes were identified. Three were novel and full-length cDNAs were obtained for two of the three. A manuscript describing the first, BRMS1, was submitted earlier this month (full-length manuscript is enclosed in the appendix). Characterization of BRMS2 is underway.

BRMS1 maps to the one arm of chromosome 11 by fluorescence in situ hybridization. Specifically, BRMS1 maps to 11q13.

KEY RESEARCH ACCOMPLISHMENTS

This section is organized according the statement of work initially proposed.

Objective #1: Map the gene(s) responsible for suppressing metastasis of MDA-MB-435 to within 5 Mb by using MMCT with radiation-deletion variants of chromosome 11

Task 1-1 (Months 1-12): Identify polymorphic markers distinguishing MDA-MB-435 and donor chromosome 11

We have identified more than 30 polymorphic markers

Task 1-2 (Months 6-18): Prepare deletion variants of chromosome 11

Several chromosome 11 donors with deletions are in hand (Section 4)

Task 1-3 (Months 7-19): Prepare microcell hybrids with radiation deletion variants

This task has been initiated. Progress has been slower than expected. (Section 4). An alternative approach using PAC and BAC transfections is being considered as an alternative. The technician responsible for this objective has been replaced by a postdoctoral fellow.

Task 1-4 (Months 8-24): Confirm hybrids actually contain added chromosome 11 material by multiple criteria

This task has been initiated with the hybrids in -hand (Section 4).

Task 1-5 (Months 12-24): Test hybrids for metastasis in orthotopic metastasis model

One of the hybrids was tested. No suppression was observed in limited experiment (Section 4, Table 2)

Task 1-6 (Months 24-48): Repeat above in independent series

Task 1-7 (Months 24-26): Map deletions in hybrids (1st set), prepare map of overlapping regions

Task 1-8 (Months 36-48): Map deletions in hybrids (2nd set), prepare map of overlapping regions

Objective #2: Stably introduce intact neo-tagged human chromosome 11 into MDA-MB-231 cells by MMCT

Task 2-1 (Months 1-6): Expand MDA-MB-231 cultures, verify pathogen-free (Mycoplasma free)

Completed first round of experiments, but none of the MDA-MB-231 variants were metastatic. Alternative strategies to obtain metastatic human breast carcinomas initiated. (Section 2)

Task 2-2 (Months 6-12): Prepare chromosome 11 hybrids

Not done, see below

Task 2-3 (Months 10-18):Confirm hybrids actually contain added chromosome 11 material by multiple criteria

Task 2-4 (Months 8-24):Test hybrids for metastasis in orthotopic metastasis model

Task 2-5 (Months 12-24): Prepare chromosome 6 and chromosome 15 hybrids, repeat metastasis study

Task 2-6 (Months 24-36):Confirm hybrids actually contain added chromosome 11 material by multiple criteria

Task 2-7 (Months 24-36): Test hybrids for metastasis in orthotopic metastasis model

Tasks 2-2 through 2-4 could not be done due to lack of appropriate models. We are attempting to obtain metastatic human breast carcinoma models in order to

accomplish this important aim. We have obtained a variant of MDA-MB-231 which is metastatic following intravenous inoculation. This model was used to characterize the metastasis suppressor gene, BRMS1, identified using specific aims 3 and 4. Work will continue in order to assess retrofit technologies to introduce portions of chromosome 11 into human breast carcinoma cells.

Objective #3: Identify metastasis-associated genes in neo11/MDA-MB-435 cells using differential display and/or subtraction hybridization

Task 3-1 (Months 6-12): Prepare cDNA library from neo11/435.B1 cells, Prepare "screening" RNA blots

Completed.

Task 3-2 (Months 6-9): Perform random primer amplification and repeat amplification for differential display

Completed.

Task 3-3 (Months 9-12): Perform "screening" Northern blots with probes from differential display

Completed.

Task 3-4 (Months 12-18): Sequence positive sequences, determine novelty, obtain full-length

Completed. Three novel cDNAs were identified. Full length cDNAs were obtained for two of the three. Despite repeated attempts to obtain full length for clone 8A3 from several cDNA libraries from several tissue sources, we are missing approximately 100 base pairs at the extreme 5' end.

Task 3-5 (Months 18-24): Repeat Northern blots with longer probes for specificity

Completed.

Task 3-6 (Months 9-18): Prepare subtraction library

See Tasks 3-1 and 3-2. Subtraction library approach was put on hold.

Task 3-7 (Months 18-30): Probe Northern blots with subtraction library

See Tasks 3-1 and 3-2. Subtraction library approach was put on hold.

Task 3-8 (Months 36-48): Obtain full-length sequence for genes expressed in subtraction library

See Tasks 3-1 and 3-2. Subtraction library approach was put on hold.

In general, this aim has been completed and we are now focusing on Objective #4 to characterize and assess the functionality of the candidates.

Objective #4: Determine whether specific genes (such as KAI-1) is a metastasis-suppressor gene in MDA-MB-435 and MDA-MB-231 cells

Task 4-1 (Months 1-6): Prepare transfectants with KAI-1

Completed

Task 4-2 (Months 6-8): Select transfectants with increased KAI-1 expression

Completed

Task 4-3 (Months 9-18): Evaluate transfectants in orthotopic metastasis assay

Completed

Task 4-4 (Months 18-48): Prepare and evaluate transfectants prepared from genes isolated in Technical Objectives 1 and 3 above.

One of the three novel cDNAs isolated by differential display, BRMS1, was transfected into MDA-MB-435 and MDA-MB-231. BRMS1 maps to human

chromosome 11 and suppresses metastasis of both human metastatic breast carcinoma cell lines. Full length BRMS2 has an isolated in transfected into both human breast carcinoma cell lines. Colonies are now being expanded for in vivo testing. Preliminary tests regarding mechanism of action of BRMS1 and brings to are underway.

REPORTABLE OUTCOMES

(Papers and Abstracts citing support from DAMD-17-1-96-6152)

FY96-97

Full-length publications

Welch, D.R. Technical considerations when studying cancer metastasis in vivo. (1997) Clinical and Experimental Metastasis 15(3): 272-306.

Lee, J.-H. and Welch, D.R. (1997) Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. Iif nternational Journal of Cancer 71: 1035-1044

Lee, J-H. and Welch, D.R. (1997) Suppression of metastasis in human breast carcinoma MDA-MB-435 cells following transfection with the metastasis-suppressor gene, KISS-1. Cancer Research 57: 2284-2287.

Yang, X., Welch, D.R., Phillips, K.K., Weissman, B.E., Wei, L.L. (1997) KAI-1, a putative marker for metastatic potential in human breast cancer. Cancer Letters 119: 149-155.

Phillips, K.K., White, A.E., Hicks, D.J., Welch, D.R., Barrett, J.C., Wei, L.L. and Weissman, B.E. Suppression of metastasis in the MDA-MB-435 model system correlates with increased expression of KAI-1 protein. Molecular Carcinogenesis 21: 111-120.

Abstracts

Welch, D.R. and Lee, J.-H. Isolation and initial characterization of the human metastasis-suppressor gene KiSS-1. Cold Spring Harbor/Frederick Cancer Research Facility Meeting on Cancer Genetics and Tumor Suppressor Genes, June 12-16, 1997.

Lee, J.-H., Goldberg, S.F., Hicks, D.J., and Welch, D.R. Suppression of human breast carcinoma MDA-MB-435 tumor growth and metastasis by KiSS-1. Proceedings of the American Association for Cancer Research (1997) 38: 545.

FY97-98

Full-length papers

Abstracts

Jaken, S., Kiley, S.C., Medina, D. Welch, D.R. Protein kinase C in mammary carcinogenesis. An Era of Hope – U.S. Army Medical Research and Materiel Command Breast Cancer Research Program (1997) 2: 411.

Lee, J.-H., Hicks, D.J., Goldberg, S.F. Welch, D.R. Suppression of human breast carcinoma MDA-MB-435 metastasis by the melanoma metastasis-suppressor gene, KiSS-1. An Era of Hope – U.S. Army Medical Research and Materiel Command Breast Cancer Research Program (1997) 2: 715.

Jaken, S., Kiley, S.C., Medina, D. Welch, D.R. Protein kinase C in mammary carcinogenesis. An Era of Hope – U.S. Army Medical Research and Materiel Command Breast Cancer Research Program (1997) 2: 411.

Lee, J.-H., Hicks, D.J., Goldberg, S.F. Welch, D.R. Suppression of human breast carcinoma MDA-MB-435 metastasis by the melanoma metastasis-suppressor gene, KiSS-1. An Era of Hope – U.S. Army Medical Research and Materiel Command Breast Cancer Research Program (1997) 2: 715.

Welch, D.R., Lee, J.-H., Miele, M.E., and Weissman, B.E. Identification of metastasis suppressor genes in human cancer. Molecular Determinants of Cancer Metastasis (1997) pp. 71-73.

Kiley, S., Goodnough, M, Clark, K., Welch, D.R., Jaken, S. Dominant negative protein kinase C-δ inhibits the metastatic progression of mammary tumor cells in vivo. Proceedings of the

American Association for Cancer Research (1998) 39: 534

Alessandrini, A. and Welch, D.R., Constitutively active MEK1 induces metastatic potential in NIH-3T3 cells. Cold Spring Harbor Meeting – Cancer Genetics and Tumor Suppressor Genes. (1998)

Fountain, J.W., Karanjawala,Z., Sridhar,A., Chen, L-L., Walker, G.J., Hayward, N.K., Welch, D.R., Rice, A., Kurera, D., Yebha, Y., Glendening, J.M., Goldberg, E.K. Localization of melanoma tumor suppressor genes on chromosome 11 using a novel method, homozygosity mapping of deletions (HOMOD) analysis. Cold Spring Harbor Meeting – Cancer Genetics and Tumor Suppressor Genes. (1998)

Alessandrini, A. and Welch, D.R. Transfection with constitutively active Mek1 confers tumorigenic and metastatic potential to NIH-3T3 cells. Clinical and Experimental Metastasis (1998)

FY98-99

Full-length papers ***

Welch, D.R. and Wei, L.L. (1998) Molecular control of breast cancer progression and metastasis. *Endocrine Related Cancers* 5: 155-196.

Kiley, S.C., Clark, K.J., Duddy, S.K., Welch, D.R. and Jaken, S. Protein kinase C δ potentiates growth in metastatic mammary cell lines. *Cancer Research* (In press).

Kiley, S.C., Clark, K.J., Goodnough, M., Welch, D.R. and Jaken, S. Dominant-negative protein kinase C-δ inhibits the metastatic progression of mammary tumor cells in syngeneic rats. *Oncogene* (In press).

Welch, D.R. In vivo cancer metastasis assays. In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Editors: Burger, M.M., Rusciano, D. and Welch, D.R. (In press)

Seraj, Md. J., Samant, R.S.., Verderame, M.J. and Welch, D.R. BrMS1, a novel human breast carcinoma metastasis suppressor gene. (Submitted)

Phillips, K.K., White, A.E., Hicks, D.J., Welch, D.R., Barrett, J.C., Wei, L.L. and Weissman, B.E. (1998) Suppression of metastasis in the MDA-MB-435 model system correlates with increased expression of KAI-1 protein. *Molecular Carcinogenesis* 21: 111-120.

Welch, D.R., Harms, J.F., Goldberg, S.F., Meehan, W.J., Seraj, M.J., Leonard, T.O., Samant, R.S., Miele, M.E., Lee, J.-H. and Hicks, D.J. Identifying and characterizing metastasis-suppressor genes in human cancer. *Biological Approaches to Cancer Therapy* 1: 32-38.

*** Copies of the manuscript/reprints/abstracts are included in the appendix. The two papers by Kiley et al are not included since they were submitted with the FY97-98 progress report.

Abstracts ***

Alessandrini, A. and Welch, D.R. Transfection with constitutively active Mek1 confers tumorigenic and metastatic potential to NIH-3T3 cells. Proceedings of the American Association for Cancer Research (1999) 40: 1312.

Verderame, M.F. and Welch, D.R. Genetic complementation of a host-dependent v-*src* mutant allele. Proceedings of the American Association for Cancer Research (1999) 40: 2453.

Barnum-Huckins, K.M., Cover, C., Robertson, G., Welch, D.R., Selmin, O., Nelson, M.A. Uniquely expressed cDNAs mediated by chromosome 1 in tumorigenicity suppressed melanoma cells. Proceedings of the American Association for Cancer

Research (1999) 40: 2122.

Rieber, M., Welch, D.R., and Rieber, M.S. Differential gene expression associated with suppression of metastatic melanoma cell spreading and proliferation on adhesion-restrictive substrates. Proceedings of the American Association for Cancer Research (1999) 40: 491.

Seraj, Md. J., Samant, R.S., Verderame, M.F., Hicks, D.J., Sakamaki, T., Hwang, C.K. Weissman, B.E., and Welch, D.R. Identification of breast cancer metastasis-suppressor genes from metastasis-suppressed chromosome 11/MDA-MB-435 hybrids. Proceedings of the American Association for Cancer Research (1999) 40: 689.

Welch, D.R. Genetic regulation of cancer metastasis. 8th Annual Meeting of the Japanese Association for Metastasis Research (1999) 8: 36.

Patent application – U.S. and Worldwide patent application has been filed for BRMS1 by Penn State University. The Office of Technology Transfer has advised that the sequence of BRMS1 not be included in this progress report

Degrees obtained that are supported by this award – None

Development of cell lines, tissue or serum repositories – Transfectant cell lines described in appended manuscripts and abstracts are available to anyone requesting them.

Informatics – None

Funding applied for based upon this work – An RO1 application will be submitted in Fall 1999 to continue this work.

Employment/research opportunities – Not applicable

CONCLUSIONS

Our preliminary data indicated the presence of one or more breast carcinoma metastasis suppressor genes on human chromosome 11. The goal of this program is to identify and begin characterizing the gene(s) responsible. Two parallel approaches were proposed – (1) introduction of smaller pieces of chromosome 11 into cells with assessment of metastatic potential; and (2) identification of differentially expressed mRNA in metastasis suppressed cells. Progress using Approach #1 has been frustrating. Therefore, we have modified the approach (i.e., assessment of retrofit technology for introduction of BAC/PAC/YAC clones into mammalian cells). While this strategy is reasonable, it has required us to learn and develop new technology within our lab.

Approach #2 has been quite productive. We have identified three novel cDNAs using differential display which are more highly expressed in the metastasis suppressed neo11/435 hybrids. Moreover, one of those candidates, BRMS1, significantly suppresses metastasis in two human breast carcinoma cell lines when transfected and constitutively expressed. BRMS1 maps to 11q13, a site commonly involved in late-stage breast carcinoma. A second candidate is being characterized and will be tested for efficacy by the end of the funding period (June 2000).

In short, we have essentially accomplished our objectives for the funding period. This has required modification of the experimental approaches initially proposed. But the general tenor of the experimental outline has been retained. We are presently completing analysis of the genes identified.

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Cheol Kyu Hwang, Ph.D.	Postdoctoral Fellow	7/1/96-6/20/97
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Deana J. Hicks, M.S.	Staff Research Assistant	12/1/96-6/30/98
Toshiyuki Sakamaki, Ph.D.	Postdoctoral Fellow	6/1/98-present

APPENDICES

AACR Abstracts

#1312 Transfection with constitutively active Mek1 confers tumorigenic and metastatic potential to NIH-3T3 cells. Alessandrini, A. & Welch, D.R. Massachusetts General Hospital, Charleston, MA 02129; Jake Gittlen Cancer Research Institute, Penn State University College of Medicine, Hershey, PA 17033-0850.

Transfection of NIH3T3 cells with constitutively active Ras results in a tumorigenic and metastatic phenotype. To identify key downstream pathways that might be involved in this process, Raf-phosphorylated serines (Ser218 and Ser222) of Mek1 were mutated to acidic residues (DS=Asp218/Ser222 and DD=Asp218/Asp222) which results in constitutively active Mek1. Transfection of DS and DD clones into NIH-3T3 or Swiss-3T3 cells increases growth in soft agar, but this growth does not correlate with Erk or Raf activity--DS lines activate Erk1/2 but yield fewer colonies. When dominant-negative Ras was introduced, Erk and Raf activities were not greatly affected. However, the same dominant negative construct introduced into v-src- or DD-transformed cells caused severe reversion of src-expressing cells, but mild reversion of DD-expressing cells. These data suggest that maintenance of in vitro transformation by Mek1 occurs through a Ras-independent pathway, and that the degree of transformation is independent of Raf1 and Erk1 activity. NIH3T3 cells transfected with DS or DD were tested for metastatic potential following i.v. injection into athymic mice. Parental cells were nontumorigenic, but DS and DD cells formed macroscopic metastases. Thus, like Ras, constitutively active Mek1 can confer both tumorigenic and metastatic potential. Further, these results refine the mechanism through which Ras could confer tumorigenic and metastatic potential--i.e., the critical determinants of tumorigenic and metastatic potential are downstream of Mek1 and may not involve Erk1/2. Support: Am Heart Assoc, CA62168, DAMD17-96-6152, Natl Fndn Cancer Res.

#2453 Genetic complementation of a host-dependent v-src mutant allele. Verderame, M.F. and Welch, D. Department of Medicine, and Jake Gittlen Cancer Research Institute, The Pennsylvania State University College of Medicine, Hershey, PA 17033.

Host-dependent alleles of v-src frequently result from mutations in the SH2 domain, whose role in transformation by v-src is poorly understood. One such host-dependent allele, v-src-F172[Delta], transforms chicken cells to a fusiform morphology but does not transform rat cells. For this allele, autophosphorylation of pp60v-src is required in fusiform-transformed chicken cells, and absent in non-transformed rat cells. To complement this SH2 domain defect, rat cells expressing v-src-F172[Delta] were transfected with several v-src alleles that are transformation-defective (td) due to mutations in different domains of the protein. Neither a td v-src allele encoding a protein that is kinase-active, not membrane localized and has no SH3 domain, nor a td allele encoding a protein that is SH2 defective and has no autophosphorylation site (but is kinase active) were able to complement v-src-F172[Delta]. In contrast, two td v-src alleles were able to effectively complement v-src-F172[Delta]: 1) v-src-K295M encoding a kinase-defective, membrane-localized src protein, and 2) v-src-G2A encoding a kinase-active src protein that is not membrane localized; both alleles retain an intact SH2 domain. Phenotypes complemented by these td alleles included morphological transformation, anchorage independent growth and tumorigenicity in syngeneic rats. These results reveal that transformation by v-src requires activation of multiple downstream signaling pathways, and provides the genetic tools to identify such pathways. Support: CA62168, DAMD17-96-6152, Natl Fndn Cancer Res.

#491 Differential gene expression associated with suppression of metastatic melanoma cell spreading and proliferation on adhesion-restrictive substrates. Manuel Rieber*, Danny R. Welch** & Mary Strasberg Rieber*. *IVIC, Tumor Cell Biology, Apartado 21827, Caracas 1020 A, Venezuela; **Jake Gittlen Cancer Research Institute, Pennsylvania State University, 500 University Drive, Hershey, Pa., 17033-0850, USA.

C8161 human melanoma metastasis is suppressed following introduction of chromosome 6 or by transfection of the KiSS-1 gene. To gain insight about the control of metastatic progression, we developed an adhesion-restrictive assay which selectively prevents spreading and proliferation of non-metastatic tumors, but is permissive for metastatic tumors. To begin elucidating the molecular processes responsible, differential gene expression was compared for cells cultured under poorly adhesive conditions. Metastatic C8161 cells exhibited increased expression of genes like [alpha]3[beta]1 integrin and 92 kDa collagenase for which expression has been associated with cell spreading and tumor invasion. In contrast, poorly metastatic cells had higher expression of genes associated with metastasis-suppression, growth arrest and/or repair of DNA damage, and down-regulation of anti-apoptotic bcl-2, cyclin D3 and 92 kDa collagenase. Decreased proliferation on suboptimal or restrictive substrates appears to be one of the mechanisms responsible for metastasis suppression in these human melanoma cells lines. Support: CA62168, DAMD17-96-6152, Natl Fndn Cancer Res.

#689 Identification of breast-cancer metastasis-suppressor candidate genes from metastasis-suppressed chromosome 11/MDA-MB-435 hybrids. Seraj, M.J., Samant, R.S., Verderame, M.F., Hicks, D.J., Sakamaki, T., Hwang, C.K., Weissman, B.E., Welch, D.R. Jake Gittlen Cancer Research Institute, Penn State College of Medicine, Hershey, PA 17033, Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC 27599.

We previously showed that introduction of a normal, neo-tagged, human chromosome 11 into the breast cancer cell line MDA-MB-435 suppressed metastasis without affecting tumorigenicity (Cancer. Res. 56: 1222, 1996). We hypothesized that this was due to a metastasis-suppressor gene(s) on 11. To identify the gene(s), differential display was undertaken. Initially, 64 differentially expressed cDNA fragments were up-regulated in the metastasis-suppressed hybrids. Subsequent screens (e.g. replicate

DD-RT-PCR, Northern blots) identified six candidate genes which were expressed >=5 fold more in neo11 hybrids than in metastatic, parental MDA-MB-435 cell s. Three of the cDNAs represented novel genes (8A3, G1A2, F5A3) while the other three cDNAs were homologous to known genes (N-acetylgalactosamine 6-sulphatase, adenine phosphoribosyltransferase and hexokinase II). Apparently full-length cDNAs of the novel genes have been isolated, introduced into mammalian expression vector and experiments to directly test their effects on metastatic potential are underway. Supported by DAMD17-96-6152, CA62168, Natl. Fndn. Cancer Res.

Japanese Association for Metastasis Research

Genetic Regulation of Cancer Metastasis, D. R. Welch, Jake Gittlen Cancer Research Institute, The Pennsylvania State University College of Medicine, Hershey, PA 17033

The ability to metastasize occurs concomitantly with nonrandom chromosomal alterations in both malignant melanoma and breast carcinoma. Among the most common aberrations occurring at the benign⇒metastatic transitions of these cancers are losses involving chromosomes 6q and 11p/11q, respectively. These observations led to the hypothesis that these chromosomal regions encode metastasis-suppressor genes. To test this, normal human, neomycin-tagged chromosomes 6 and 11were introduced into C8161 human melanoma and MDA-MB-435 breast carcinoma cell lines by microcell-mediated transfer. *In vivo* metastasis assays involving orthotopic injection into athymic mice showed that chromosome 6 suppressed melanoma, but not breast carcinoma, metastasis. In contrast, chromosome 11 suppressed breast, but not melanoma, metastasis. Tumorigenicity was unaffected in both cases.

To identify the genes responsible, differential display and subtractive hybridization were used. Comparison of nonmetastatic chromosome 6/C8161 hybrids to metastatic C8161 cells revealed several qualitative and quantitative differences. The best characterized is KiSS-1. Expression was evaluated by Northern blot. KiSS-1 was not detectable in any metastatic melanomas, but was expressed in normal human melanocyte and benign radial growth phase cultures. Transfection of five human melanoma cell lines (C8161, MelJuSo, M24met, A375M, MeWo-3S5) and one breast (MDA-MB-435) resulted in significant (>50%) suppression of metastasis without impacting tumorigenicity. So, KiSS-1 satisfied the criteria to be called a metastasis-suppressor gene. Surprisingly, KiSS-1 mapped to 1q32 and its expression appears to be regulated by genes encoded at 6q13-q23. The regulator(s) has not yet been identified. Genomic KiSS-1 has recently been cloned and analysis of the 5'-UTR is currently underway.

The KiSS-1 cDNA is predicted to encode a novel protein of Mr ≈15.4 kDa which is mostly hydrophilic, and which contains no obvious signal or nuclear localization sequences. The amino acid sequence includes several motifs surrounding Ser/Thr/Tyr residues indicative of KiSS-1 being a phosphoprotein. Most putative interactors thus far identified by yeast two-hybrid screening have known functions consistent with the hypothesis that KiSS-1 is a cytoplasmic protein involved in cell signaling (S.F. Goldberg, J.E. Hopper & D.R. Welch, unpublished).

In a protocol analogous to the one used to identify KiSS-1, three novel and four known cDNAs have been isolated from chromosome 11/MDA-MB-435 hybrids. All of these cDNAs are expressed at ≥5-fold higher levels in the hybrids than the metastatic parental cells. Preliminary data will be presented.

In addition to the above findings supporting the existence of cancer type-specific metastasis suppressor activities, metastasis-enhancing signals have been identified. Using a panel of differentially metastatic rat mammary adenocarcinoma cell lines, PKCδ expression was found to increase with metastatic potential. Upregulation of PKCδ in poorly metastatic clones and downregulation using a dominant negative construct in highly metastatic clones resulted in increased and decreased metastatic potential, respectively.

Taken together, the results show that metastasis in melanoma and breast share some, but not all, regulatory mechanisms.

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APPENDICES:

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BRMS1, a suppressor of metastasis in human breast carcinoma
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***BRMS1*, a suppressor of metastasis in human breast carcinoma**

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Abbreviations:

BAC, bacterial artificial chromosome; *BRMS1*, Breast cancer metastasis suppressor-1; CMF-DPBS, calcium- and magnesium-free Dulbecco's phosphate buffered saline; DAPI, 4',6-diamidino-2-phenylindole; DD-RTPCR, Differential display; DME/F12, mixture (1:1) of Dulbecco's-modified minimum essential medium and Ham's F-12 medium; FISH, fluorescence in situ hybridization; HBSS, Hank's balanced salt solution; PAC, P1 artificial chromosome; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TE, 0.125% trypsin, 2 mM EDTA solution in CMF-DPBS; TTBS, Tris-buffered saline.

Key Words: Differential display, chromosome 11,

Running title: *BRMS1*, a cancer metastasis suppressor gene

Figures: 5

Tables: 2

Abstract

Introduction of normal human chromosome 11 reduces the metastatic capacity of MDA-MB-435 human breast carcinoma cells without affecting tumorigenicity [Phillips *et al.* (1996) *Cancer Research* 56: 1222-1227]. This suggests the presence of one or more metastasis suppressor genes on human chromosome 11. Differential display was undertaken to identify mRNAs with increased levels of expression in neo11/435 hybrids compared with their metastatic counterparts. Six cDNA fragments were consistently differentially expressed in replicate amplifications and RNA analyses at levels at least 5-fold greater in metastasis-suppressed neo11/435 hybrids. Three of the six candidates were homologous to known cDNAs (N-acetylgalactosamine-6-sulfate-sulphatase, adenine phosphoribosyltransferase and hexokinase II). The remaining three had minimal homology to known sequences or ESTs. In this paper, we describe the isolation and functional characterization of a full-length cDNA for one of the novel genes, designated *BRMS1* (**Br**east-cancer **M**etastasis **S**uppressor **1**), which maps to human chromosome 11q13.1-q13.2 by fluorescence *in situ* hybridization. *BRMS1*-transfected MDA-MB-435 and MDA-MB-231 cells form significantly fewer metastases in athymic mice than parental or vector-only controls in an expression-dependent manner, demonstrating that *BRMS1* is a metastasis-suppressor. Like the neo11/435 hybrids, *BRMS1* transfectants remain tumorigenic. The mechanism by which *BRMS1* suppresses metastasis is not fully elucidated, but does not appear to involve upregulation of Nm23-H1, KiSS1 or KAI1 metastasis-suppressor genes. BRMS1 predicted protein sequence contains regions with homology to DNA binding domains, coiled-coil, leucine zipper, nuclear localization and some consensus phosphorylation sites. These homologies suggest that BRMS1 may function in a signaling cascade as a transcription factor.

Introduction

Worldwide mortality from breast carcinomas is expected to exceed 300,000 people in 1998 {Landis, Murray, et al. 1999 13573 /id}. When breast carcinoma cells are confined to breast tissue, long-term survival rates are high. However, when tumor cells disseminate to and colonize secondary sites, cure rates drop significantly. Likewise, quality of life for patients with Stage IV (metastatic) disease is significantly worse than for those with Stage I (local) carcinoma {Hortobagyi & Piccart-Gebhart 1996 7522 /id}. Thus, decreased morbidity and mortality will depend upon prevention of and/or effective treatment of metastatic disease. To that end, understanding the biologic, biochemical and genetic mechanisms underpinning tumor cell invasion and metastasis will be required {Fidler 1999 15017 /id}.

To metastasize, neoplastic cells dissociate from the primary tumor, enter a circulatory compartment (typically lymphatics or blood vasculature), survive transport, arrest, exit the circulation and finally

proliferate at a discontinuous site in response to local growth factors. Unless cells accomplish every step in the metastatic cascade, metastases cannot develop. The process is highly inefficient, i.e., less than 0.1% of cells entering the vasculature form clinically detectable secondary tumors {Weiss, Dimitrov, et al. 1985 390 /id} {Weiss & Ward 1983 6355 /id} {Luzzi, MacDonald, et al. 1998 12361 /id}. It is clear that at each step of the metastatic cascade, multiple genes and proteins are involved. Because inappropriate movement of cells with subsequent colonization of secondary sites inherently implies that some genes are either mutated or aberrantly regulated, it follows that identifying and manipulating metastasis-regulatory genes could lead to decreased efficiency of the metastatic process and better systemic control of neoplasia. Moreover, it must be emphasized that each of the genetic defects responsible for developing metastatic potential is superimposed over those already involved in the genesis of a tumor {Dear & Kefford 1990 314 /id} {Welch & Goldberg 1997 6584 /id} {Welch & Wei 1998 11672 /id}. In the case of negative regulators, the distinction between tumor suppressors and metastasis suppressors is critical. While the former will block tumorigenicity, tumor suppressors also block metastasis since tumor formation is a prerequisite to metastasis. However, metastasis suppressors will block spread without stopping tumor formation. As such tumor suppressors and metastasis suppressors represent distinct therapeutic targets. In addition, just as there are patterns of association for various oncogenes and tumor suppressor genes in the ontogeny of various tumor types, different metastasis suppressors and metastasis promoters will also very likely have unequal involvement in tumors of diverse histologic origin.

To study the genetics of metastasis by human infiltrating ductal carcinoma of the breast, we have taken advantage of an extensive literature showing high frequency non-random chromosomal alterations associated with different stages of tumor progression (reviewed in {Welch & Wei 1998 11672 /id}). Specifically, some chromosomal gains/losses occur concomitant with acquisition of invasive and metastatic competency. Among the most common chromosomal alterations in late-stage breast carcinoma are losses involving the short and long arms of chromosome 11. The temporal association of chromosomal alterations with phenotypic changes leads to the hypothesis that those regions would harbor metastasis-controlling genes. To determine the effect of chromosome 11 on breast carcinoma metastasis, we introduced a normal human chromosome 11 into metastatic MDA-MB-435 cells using microcell-mediated chromosome transfer. Hybrid cell clones, abbreviated neo11/435, were suppressed for metastasis but tumorigenicity was unaffected {Phillips, Welch, et al. 1996 5718 /id}. Based upon these results, we refined the hypothesis to predict that a breast cancer metastasis-suppressor gene is encoded on chromosome 11. To identify the gene(s) responsible, differential display {Liang & Pardee 1992 2358 /id} {Liang, Averboukh, et al. 1993 2359 /id} was used to compare gene expression in metastasis-

competent with metastasis-suppressed MDA-MB-435 variants. We report here the identification, isolation and characterization of one of those genes, *BRMS1*, which suppresses metastasis and maps to human chromosome 11q13.1-q13.2.

Materials and Methods

Cell Lines. MDA-MB-435 and MDA-MB-231 are human estrogen receptor- and progesterone receptor-negative, metastatic, infiltrating ductal breast carcinoma cell lines {Price, Polyzos, et al. 1990 246 /id} {Price 1996 6298 /id} {Cailleau, Olive, et al. 1978 2282 /id} {Cailleau, Young, et al. 1974 2334 /id}. Both cell lines form progressively growing tumors when injected into the mammary fat pads of immunocompromised mice. MDA-MB-435 cells develop macroscopic metastases in the lungs and regional lymph nodes by 10-12 weeks post-inoculation, but rarely metastasize following direct injection into the lateral tail vein. The opposite pattern exists for MDA-MB-231 in athymic mice. MDA-MB-435 cell clones into which a normal, neomycin-tagged human chromosome 11 had been introduced by microcell-mediated transfer (designated neo11/435) are suppressed at least 75% for metastasis from the mammary fat pad {Phillips, Welch, et al. 1996 5718 /id}.

BRMS1 transfectants (see below) were derived following transfection of full-length *BRMS1* cDNA in constitutive mammalian expression vector, pcDNA3 (Invitrogen, San Diego CA). All cell lines were cultured in a 1:1 mixture of Dulbecco's-modified minimum essential medium and Ham's F-12 medium (DME/F12) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% in non-essential amino acids, 1.0 mM sodium pyruvate, but no antibiotics or antimycotics. Transfected cells and neo11/435 hybrids also received 500 μg/ml geneticin (G-418, Life Technologies, Inc. [GIBCO-BRL], Gaithersburg, MD, USA). Cell cultures were maintained on 100 mm Corning tissue culture dishes at 37° C with 5% CO_2 in a humidified atmosphere. When cultures reached 80-90% confluence, they were passaged using a solution of the 0.125% trypsin, 2 mM EDTA (TE) in Ca^{+2}/Mg^{+2}-free Dulbecco's phosphate buffer saline (CMF-DPBS). *BRMS1*-transfected MDA-MB-435 cells acquired an acute sensitivity to trypsin; so, cultures were thereafter passaged using 2 mM EDTA solution in CMF-DPBS. Cells could be routinely split at ratios of 1:10-1:30. *In vitro* doubling times for all cells were typically between 24-36 hr. MDA-MB-435 and MDA-MB-231 cells were used between passages 119-139 and 161-166, respectively. Hybrid clones and transfectants were used before passage 11 in all cases to minimize the impacts of clonal diversification and phenotypic instability {Welch & Tomasovic 1985 90 /id}. For all functional and biological assays, cells between 70-90% confluence were used with viability >95%. All the lines were routinely checked and found to be negative for *Mycoplasma spp*. contamination using the GenProbe method (Fisher Scientific, Pittsburgh, PA).

Cell line nomenclature was developed to identify the origin and nature of each cell line as unambiguously as possible. Single-cell clones are identified by the cell line preceding a "." followed by a clonal designation while uncloned, populations are identified by a "–" after the parental cell line name. Microcell hybrids are identified by the tagged chromosome number followed by a "/" (e.g., neo11/435.A3 is single cell clone A3 derived following microscope mediated transfer of chromosome 11 into MDA-MB-435). Where appropriate, numbers in parentheses following the cell line designation indicate the number of subcultures following cloning or establishment of the cell line. Numbers preceded by a "TE" indicate that the cells were passaged in a mixture of trypsin-EDTA . Numbers preceded by a "P" indicate the cells were passaged using EDTA alone. In many figures, MDA-MB-435 cells are abbreviated "435" and MDA-MB-231, "231" for space considerations.

Transfections. BRMS1 was cloned into the constitutive mammalian expression vector, pcDNA3. To detect BRMS1 protein expression, a chimeric molecule was also constructed with an N-terminal epitope tag (SV40T epitope 901, see below {Fu, Bonneau, et al. 1996 15116 /id} {Kierstead & Tevethia 1993 15117 /id}). Epitope-tagged and native *BRMS1* plasmids as well as pcDNA3 vector only were transfected into MDA-MB-435 and MDA-MB-231 cells by electroporation (BioRad Model, Hercules, CA; 220V, 960μFd, ∞Ω). Briefly, cells (0.8 ml; $1x10^7$ cells/ml) from 80% confluent plates were detached using a 2 mM EDTA solution. Plasmid DNA (10-40 μg) was placed onto ice for 5 min before electroporation followed by 10 min afterward before plating onto two 100 mm tissue culture dishes. One day later, transfectants were selected by addition of G-418. Single cell clones were isolated by limiting dilution in 96 well plates. Stable transfectants of *BRMS1* were assessed for their expression of transcripts by Northern blotting and, as appropriate by Western blotting.

Differential Display (DD-RTPCR). To identify differences in mRNA expression between metastatic and nonmetastatic neo11/435 hybrid cells, a differential display approach was undertaken. The methods used involved minor modifications of the method described by Liang and Pardee {Liang & Pardee 1992 2358 /id} {Liang, Averboukh, et al. 1993 2359 /id} as available in the Delta™ Differential Display kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). Briefly, poly A^+-enriched mRNA was isolated using oligo dT cellulose (Invitrogen Corp., USA) as previously described {Ausubel, Brent, et al. 1998 11068 /id}. Also, to minimize the impact of clonal heterogeneity, equal parts mRNA from three neo11/435 hybrid clones (neo11/435.A3 (TE5), neo11/435.B1 (TE4), neo11/435.D1 (TE10)) were used. RT-PCR products were resolved in a denaturing 5% polyacrylamide /8 M urea gel. Dried gels were then exposed to X-ray film (Reflection™, DuPont-NEN, Boston, MA, USA) for 2 to 12 h at room temperature and/or –70°C. Unique bands from metastasis-suppressed neo11/435 lanes that were reproducibly obtained in two

independent DD-RTPCR reactions and the corresponding areas from metastatic MDA-MB-435 lane were excised from the dried gel. cDNAs were eluted by soaking the gel slices in H_2O (100 µl) for 10 min and boiling for 15 min before ethanol precipitation in the presence of 3 M sodium acetate and 50 µg glycogen as a carrier. They were then re-dissolved in water (10 µl). Aliquots (2 µl) were subjected to another round of PCR using the same set of primers and conditions except no radioisotope was included in the reaction mixture. The amplified PCR products were analyzed on a 2% agarose gel. To minimize risks of false positives, failure to amplify a product from an irrelevant region of the gel was also a criterion before proceeding to the Northern blot analyses.

Differentially expressed cDNAs and the primers used for the initial DD-RT-PCR reactions were: *BRMS1* [P9/P9]; F5A3 [P9/T5], 8A3 [P3/T4], adenine phosphoribosyltransferase [P1/T9]; N-acetyl-galactosamine-6-sulfate sulfatase [P6/P6]; hexokinase II [P10/T8].

P1: 5' - ATTAACCCTCACTAAATGCTGGGGA - 3'

P3: 5' - ATTAACCCTCACTAAATGCTGGTGG - 3'

P6: 5' - ATTAACCCTCACTAAATGCTGGGTG - 3'

P9: 5' - ATTAACCCTCACTAAATGTGGCAGG - 3'

P10: 5' - ATTAACCCTCACTAAAGCACCGTCC - 3'

T4: 5' - CATTATGCTGAGTGATATCTTTTTTTTCA - 3'

T5: 5' - CATTATGCTGAGTGATATCTTTTTTTTCC - 3'

T8: 5' - CATTATGCTGAGTGATATCTTTTTTTTGC - 3'

T9: 5' - CATTATGCTGAGTGATATCTTTTTTTTGG - 3'

Northern Blot Hybridization. Poly A^+-enriched mRNAs (2 µg) were size-separated on denaturing 1% agarose formaldehyde gels before transferring onto a positively charged Hybond-N nylon membrane (Amersham Life Sciences Inc., Arlington Heights, IL, USA) using the Turboblotter system (Schleicher & Schuell, Keene, NH, USA) and fixation by UV cross-linking. Differentially expressed, gel-purified PCR products were radiolabeled by [α-^{32}P]dCTP (Dupont-NEN) using the random primer labeling method and used as probes. All prehybridization and hybridizations were carried out using ExpressHyb Solution (Clontech) according to the manufacturer's recommendations, except that washes were performed at 55°C rather than 50°C. The membranes were exposed to Kodak BioMax MR x-ray film. Equal loading

and transfer efficiency were assessed by hybridizing the blots with human GAPDH cDNA (Pst1/Kba1 780 bp fragment of ATCC57090/ATCC57091 in pBR322).

Novel candidates exhibiting differential expression between the neo11/435 and MDA-MB-435 cells were then assayed for mRNA expression patterns in multiple human tissues using a human mRNA master dot blot (Clontech) using the same PCR product probes described above and quantified by phosphorimage analysis. This information was used to identify the best library from which to obtain full-length cDNAs (see below). Once full-length cDNAs were obtained, differential expression was verified in the human breast carcinoma cell lines and multiple tissue northern blots (Clontech).

Cloning of differentially expressed fragments. Only cDNA clones that consistently expressed at least 5-fold more in the neo11/435 hybrids were chosen for further analysis. PCR fragments were subcloned into the pCR2.1 TA cloning vector (Invitrogen) and sequenced using the dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Nucleotide sequences were compared to known genes in the Genbank, EMBL and EST databases using the computer program BLAST.

cDNA library screening. For isolation of the full length cDNA, a normal adult human kidney cDNA library constructed in λTripIEx vector (Clontech) was screened using the [α^{32}P] dCTP-labeled *BRMS1* PCR product as a probe. Briefly, unamplified library (1 x 10^4 pfu; 1 µl; 1:5 dilution) was used to infect 200 µl of *E. coli* XL1 blue MRF' culture ($O.D._{600}$ = 2.00) grown in LB medium supplemented with $MgSO_4$ (10 mM) and maltose (0.2%). The infected culture was mixed with 2.5 ml melted LB/$MgSO_4$ top agar and poured onto 90 mm LB/$MgSO_4$ plate pre-warmed to 37^0 C. The plates were then incubated at 37^0 C for 14 hr. Plaques were lifted using plaque screen hybridization transfer membranes (Dupont-NEN). The membranes were treated 10 min each with denaturation solution (0.5 N NaOH, 1.5 M NaCl) followed by neutralization solution (0.5 M Tris-Cl pH 8.0, 1.5 M NaCl). Dried membranes were fixed by baking at 80°C for 2hr. After three rounds of screening, the λTripIEx lysate from the confirmed positive plaques was transduced into *E. coli*BM 25.8, to promote *Cre* recombinase- mediated release and circularization of the pTripIEx at the *loxP* site. The recombinant pTripIEx were then maintained in *E. Coli* XL1 blue MRF'. The isolated cDNA clones were sequenced and compared with *BRMS1* and scanned for a continuous open reading frame preceded by a Kozak sequence {Kozak 1984 5598 /id}.

Southern Blot Hybridization. The presence of *BRMS1* gene in various eukaryotic species other than human was examined by Southern blot hybridization. Full-length *BRMS1* cDNA was used to probe a Zoo-blot (Clontech) that had genomic DNA from nine eukaryotic species (chicken, cow, dog, human, monkey, mouse, rabbit, rat, yeast) digested with *Eco*Rl, resolved on a 0.7% agarose gel, transferred to a

charge-modified nylon membrane by capillary transfer and fixed by UV irradiation.

In vitro transcription and translation. To demonstrate that a full-length cDNA would indeed produce protein, *BRMS1* was cloned directionally into pcDNA3 (Invitrogen). The resultant plasmid construct was used for TNT® T7 Quick Coupled Transcription/Translation System (Promega Corp., Madison, WI, USA).

Chromosomal localization of BRMS1. BRMS1 cDNA was used to screen bacterial artificial chromosome (BAC) and P1 artificial chromosome (PAC) libraries at Genome Systems, Inc. (St. Louis, MO, USA). BAC clones 412(n24) and 536(h18) harbored *BRMS1* and was confirmed by direct sequencing (R.S. Samant and D.R. Welch, unpublished observations). DNA was isolated and labeled with digoxigenin dUTP by nick translation and combined with sheared human DNA before hybridization to metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood leukocytes in a solution containing 50% formamide, 10% dextran sulfate and 2X SSC. Specific hybridization signals were detected by exposing the hybrid cell lines to anti-digoxigenin antibodies followed by counterstaining with DAPI. Specific labeling along the proximal long arm of a group C chromosome, which was subsequently confirmed to be chromosome 11 based upon co-hybridization with genomic probes known to map to 11p15 and 11cen. Measurements of 71/80 specifically labeled chromosomes 11 in metaphase spreads demonstrated that *BRMS1* is located at a position which is 19% of the distance from the centromere to the telomere of chromosome 11q. This corresponds to band 11q13.1-q13.2 (data not shown).

Immunoblotting. A monoclonal antibody developed against the 901 epitope of the SV40T antigen (amino acids 684-698, developed in the laboratory of Dr. Satvir Tevethia, Department of Microbiology and Immunology, Penn State University College of Medicine {Fu, Bonneau, et al. 1996 15116 /id} {Kierstead & Tevethia 1993 15117 /id}) was generously provided. BRMS1 with the 901 epitope fused in-frame to the N-terminus was cloned into pcDNA3 before transfection into MDA-MB-435 and MDA-MB-231 and single cell cloning as above.

BRMS1 expression was determined by collecting total protein of 70-90% confluent cell cultures. Following aspiration of medium, plates were rinsed three times with CMF-DPBS before addition of 1 ml lysis buffer (50 mM Tris-HCl, pH 6.8; 2% β-mercaptoethanol, 2% SDS). Lysates were centrifuged at 10,000 x g at 4°C for 15 min to remove insoluble material. Protein concentration was determined using the Bradford method {Bradford 1976 2212 /id}. Protein (20-30 μg per lane) was mixed with 5X loading buffer (50% glycerol, 1.5% bromphenol blue) and separated by 12.5% SDS-PAGE {Laemmli 1970 1180

/id}. Proteins were transferred to PolyScreen® membrane (NEN-Dupont) by semi-dry transfer (5.5 mA/cm^2, 20V, 30 min). Proteins were fixed by air drying for 15 min at room temperature. Membranes were then wetted in absolute methanol, rinsed in distilled water and blocked in a TTBS solution (0.05% Tween-20; 20 mM Tris, 140 mM NaCl, pH 7.6) containing 5% dry nonfat milk for 1 hr. The 901-tagged BRMS1 was detected using a 1:5000 dilution of mouse anti-901 ascites for 1 hr at room temperature under constant agitation. Membranes were then washed with TTBS and probed with a 1:10,000 dilution of sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham-Pharmacia Biotech, Buckinhamshire, UK) in a solution of 5% nonfat dry milk/TTBS for 1 hr at room temperature before washing in TTBS. Bound secondary antibodies were detected using ECL™ (Amersham-Pharmacia Biotech) for 30 sec to 10 min. Similar protocols were used to detect Kai1 (rabbit anti-KAI1, Santa Cruz; 1:5000); Nm23 (rabbit anti-human, NeoMarkers, Fremont, CA, 1:5000) and E-cadherin (rabbit anti-E-cadherin, Transduction Laboratories, Lexington, KY, 1:3000) using donkey anti-rabbit IgG HRP conjugate as a secondary antibody at a titre of 1:5000. Some of the blots were stripped and re-probed using a solution of 200 mM glycine, 50 mM potassium acetate and 0.2% β-mercaptoethanol, pH 4.5.

Metastasis Assay. Immediately prior to injection, cells (7-11 passages following transfection) at 80-90% confluence were detached with a 2 mM EDTA solution. Cells were washed, counted on hemacytometer, and resuspended in ice-cold HBSS to a final concentration of 2.5 x 10^6 cells/ml for MDA-MB-231 cells and 1 x 10^7 cells/ml for MDA-MB-435 cells. MDA-MB-231 cells and derivatives (0.5 x 10^6 in 0.2 ml) were injected intravenously into the lateral tail vein of 3-4 wk old, female athymic mice (Harlan Sprague-Dawley, Indianapolis, IN) using a 27 gg needle affixed to a 1 cc tuberculin syringe. Mice were killed 4 weeks post-injection and examined for the presence of metastases. Lungs were removed, rinsed in water and fixed in Bouin's solution before quantification of surface metastases as previously described {Welch 1997 7126 /id}.

Similar procedures were used for the spontaneous metastasis assay using MDA-MB-435 cells, except that 1 x 10^6 cells (0.1 ml) were injected into exposed axillary mammary fat pads of anesthetized 5-6 wk old, female athymic mice {Welch 1997 7126 /id}. Tissue from representative metastatic lesions was preserved for histologic analyses. Sections (4-6 μm) were prepared by fixation in Bouin's solution or neutral buffered formalin followed by dehydration, paraffin embedding, sectioning and staining with hematoxylin and eosin.

Tumor size was measured weekly by taking orthogonal measurements and was expressed as mean tumor diameter. Mean tumor diameter was calculated as described {Welch 1997 7126 /id} by used of the

following the equation: $\sqrt{(diameter_x \times diameter_y)}$,

where x and y are orthogonal measurements of the locally growing tumor.

After the mean tumor diameter reached 1.5 – 2.0 cm, tumors were surgically removed under Ketamine: Xylozine (80-85 mg/kg:14-16 mg/kg) anesthesia and the wounds closed with sterile stainless steel clips. Four weeks, later, mice were killed and visible metastases were counted {Welch 1997 7126 /id}. Lung tissues were handled as above. Metastases were also observed in the ipsilateral and contralateral axillary lymph nodes of control mice. Occasional recurrences developed at the site of tumor removal but the presence of hematogenous metastases did not necessarily correlate with presence of recurrent tumor.

Animals were maintained under the guidelines of the National Institutes of Health and the Pennsylvania State University College of Medicine. All protocols were approved by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*.

Statistical analyses. The number of lung metastases was compared for *BRMS1* transfectants and corresponding parental and vector-only transfected MDA-MB-435 and MDA-MB-231 cells. For experimental metastasis assays, one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference post-hoc test was used. For spontaneous metastasis assays, a Kruskal-Wallis ANOVA of ranks procedure was used with Dunn's post-hoc test. Calculations were performed using SigmaStat statistical analysis software (Jandel Scientific, San Rafael, CA). Statistical significance was designed as $P \leq 0.05$ using two-tailed tests.

Results

To identify the gene(s) responsible for metastasis suppression following introduction of chromosome 11 into MDA-MB-435 cells {Phillips, Welch, et al. 1996 5718 /id}, differential display was used. Parallel DD-RTPCR reactions were performed initially with individual neo11/435 hybrid clones and parental MDA-MB-435; however, none of the differences held up to more rigorous scrutiny (unpublished observations). We reasoned that part of the problem could have been heterogeneity within the parental population (i.e., presence of both metastatic and nonmetastatic variants). To minimize this complication, equal mixtures of RNAs from three neo11/435 clones were mixed and differential display was repeated.

To reduce further the chances of proceeding with irrelevant cDNAs, we validated findings at several intermediate steps during the experiment. In short, all RT-PCR reactions were replicated using independent samples. If PCR products could not be amplified with the same primers, they were no longer

pursued. Once PCR products were validated, they were used as probes to examine differential expression in Northern blots using progressively more extensive series of cell line mRNAs. Finally, differential expression of at least 5-fold was chosen as a criterion for proceeding to the next steps of obtaining full-length cDNAs and functional studies.

Initially, 64 cDNA fragments were detected as upregulated in the neo11/435 hybrids. A representative result is shown in **Figure 1A**.. Only 18 bands reproducibly amplified in replicate RT-PCR reactions (data not shown). Of these, 6 fragments exhibited ≥5-fold higher mRNA expression in neo11/435 hybrid cell clones as detected by Northern blotting and quantified by phosphor image analysis (**Figures 1B and 1C**). None of the PCR products detected mRNAs expressed exclusively in the metastasis-suppressed cells. The six cDNA inserts were sequenced and homology to known genes and ESTs was assessed by comparing with the GenBank / EMBL / DDBJ / PDB combined database (**Table 1**). Three of the cDNAs were highly homologous to known human genes for the regions that overlapped (N-acetylgalactosamine-6-sulphatase, adenine phosphoribosyltransferase and hexokinase II). The remainder were novel and became priorities for further study.

In order to obtain full-length cDNA clones, each of the PCR products was used to probe multi-tissue RNA blots. Phosphor image analysis of RNA dot blots showed that all three novel cDNAs were most highly expressed in kidney, pancreas, spleen and testis (data not shown). A human kidney library was chosen for isolation of full-length cDNAs. Here we report on the isolation and functional characterization of one of these novel cDNAs, *BRMS1*.

The nucleotide sequence of the *BRMS1* cDNA initially revealed no significant homologies to any known genes, ESTs or proteins deposited in the databases. However, subsequently a deposited partial sequence (Accession number AL050008; hypothetical human protein sequenced by AGOWA within the cDNA sequencing consortium of the German Genome Project) had 91% homology at the predicted amino acid level for the region bounded by amino acids 60-244. Regions of *BRMS1* cDNA showed numerous homologies to short ESTs isolated from fetal liver and spleen as well as the human tumor cell line HeLa, consistent with the wide tissue expression observed in the multi-tissue Northern blots. *BRMS1* was detected in multiple species at the DNA level, but was most abundant in yeast, human, mouse, rat, rabbit and cow in Southern blots.

The *BRMS1* cDNA sequence was submitted to GenBank as a novel human gene with an accession number of AF159141. Computerized analysis shows that *BRMS1* cDNA length is 1485 base pairs with the largest open reading frame of 741 base pairs (from nucleotides 122 to 862 (**Figure 2**)). *BRMS1*

encodes a novel protein of 246 amino acids (Mr ≈ 28.5 kDa), a result confirmed using *in vitro* transcription and translation (data not shown). Fluorescence *in situ* hybridization mapping places the location of *BRMS1* gene at human chromosome 11q13.1-q13.2.

The predicted amino acid sequence of BRMS1 was analyzed for structural and sequence homologies in order to obtain clues regarding mechanism of action. While the protein is novel, several regions show homology to known motifs using the algorithms associated with the search engines mentioned. Using PROSITE (http://www.genebio.com/prosite.html), several putative phosphorylation sites for cAMP/cGMP ($[R/K]_2$-x-[S/T]; amino acids 55-58 and 240-243), protein kinase C ([S/T]-[R/K]; amino acids 111-113, 147-149, 190-192, 200-202) and casein kinase II ([S/T]-x_2-[D/E]; amino acids 19-22, 30-33, 37-40, 39-42, 41-44, 46-49) were detected. PSORT II (http://www.psort.nibb.ac.jp:8800) identified two putative nuclear localization sequences (amino acids 198-205 and 239-245) and predicted at 60.9% probability that BRMS1 would localize to the nucleus. In addition, BRMS1 contains two coiled-coil (amino acids 51-81 and 147-180) motifs and several imperfect leucine zipper (L-x_6-L-x_6-L-x_6-L) motifs at amino acids 67-88, 131-152, 138-159, 153-174 and 160-181. No signal peptide motifs were identified but there was a potential endoplasmic reticulum retention sequence at amino acids 243-246. These findings were also confirmed using the http://www.expasy.ch search engine. There are four cysteine residues within the BRMS1 protein, allowing for the possibility of intra- and inter-protein disulfide linkages.

Full-length *BRMS1* cDNA was used to probe mRNA blots prepared from parental MDA-MB-435 and a panel of neo11/435. Again, the metastasis-suppressed lines displayed higher expression **(Figure 1B)**. Expression in a variety of normal tissues was observed using a multi-tissue Northern blot, with highest expression in kidney, pancreas, spleen, testis, skeletal muscle and peripheral blood lymphocytes, moderate expression in heart, liver, placenta and intestines, and low levels in brain, lung and thymus **(Figure 1C)**. A single 1.5 kb band was detected in all tissues, suggesting that *BRMS1* mRNA is present as a single form and not commonly as alternatively spliced species.

To assess the effect of *BRMS1* on breast carcinoma tumorigenicity and metastasis, *BRMS1* was transfected into two metastatic human breast carcinoma cell lines, MDA-MB-435 and MDA-MB-231 **(Figure 3A)**. In order to more readily assess protein expression. BRMS1 was epitope-tagged using the SV40T901 epitope and Western blots were used to assess protein levels in the transfectants. mRNA message levels generally correlated well with BRMS1 protein level in these cells **(Figure 3B, top panel)**. Concomitantly, MDA-MB-231 transfectants were tested for expression of the known metastasis-suppressor genes, Nm23, KAI1, E-cadherin (immunoblot, **Figure 3B, lower panels**) and KiSS1 (Northern blot, not shown). These experiments were done to evaluate whether the metastasis-suppressing

effect of *BRMS1* could be explained by modulation of expression of other known metastasis-suppressor genes. Expression patterns of these suppressor genes did not correlate with *BRMS1* mRNA or protein expression, suggesting that BRMS1 activity is not dependent upon the expression of these other genes. However, insufficient data exist to rule out interactions among BRMS1 and the other metastasis suppressor genes. MDA-MB-231 cells have no detectable E-cadherin protein in Western blots. It is worth noting that the sequence of the other metastasis suppressors has not been determined in the MDA-MB-231 cells.

Clones representing low, medium and high BRMS1 protein expression were chosen for functional studies. In cell culture, *BRMS1* transfectants generally exhibited a more flattened morphology (**Figure 4A**) and tended to aggregate more readily following detachment. However, these properties were not evident in every clone isolated. *in vitro* growth rates for *BRMS1* transfectants were similar to parental and vector-only transfectants. Surprisingly, MDA-MB-435 transfected with *BRMS1* acquired an acute sensitivity to trypsin (data not shown). Whereas parental cells were routinely passaged using TE, the *BRMS1* transfectants died when exposed to even low concentrations of trypsin. Therefore, subsequent cultures were handled using EDTA to detach the cells from the substrata.

BRMS1-transfectants were assessed for tumorigenicity and metastasis following injection into athymic mice. Growth of *BRMS1*-transfected MDA-MB-435 tumors in the mammary fat pad was generally slower than in the parental and/or vector controls. In general, once the tumors began to grow, their size was approximately one week behind the parental, metastatic populations. Representative *in vivo* growth curves are shown in **Figure 4B**. In general, the *in vivo* morphologies of MDA-MB-435 and *BRMS1*-transfectants were similar, except that the latter exhibited fewer fibrous bands in the stromal compartment of the tumors (**Figure 4C**). While tumorigenicity was unaltered, the incidence and number metastasis to lung and regional lymph nodes was significantly (P=0.004) suppressed in the MDA-MB-435 *BRMS1*-transfectants (**Table 2**). Parental and vector-only transfectant cells formed axillary lymph node and lung metastases in 100% of the mice injected. In the *BRMS1*-transfectants, the incidence dropped by 50-90%. Of the metastases that formed, all were significantly smaller than the parental lesions at a comparable time following injection. Even if the the metastases were given more time to grow, most did not develop into grossly visible lesions. BRMS1 expression was still detectable in the BRMS1-transfected MDA-MB-435 locally growing tumors. Using the model of intravenous inoculation of MDA-MB-231 cells, similar suppression of metastasis was observed (**Figure 5**). Comparison of the level of BRMS1 protein with metastatic potential suggests that the effect is dose-dependent.

Discussion

To identify human breast cancer metastasis suppressor genes, we took advantage of observations identifying numerous nonrandom chromosomal changes during breast carcinoma progression (reviewed in {Welch & Wei 1998 11672 /id}). Although difficult to assign specific chromosome structural abnormalities to particular stages of breast cancer; some karyotypic changes commonly occur during early-stage breast cancer — 8p, 13q, 16q, 17p, 17q and 19p — while others typically occur later in breast cancer progression — 1p, 1q, 3p, 6q, 7q, 11p, and 11q. If the latter changes occur concomitant with acquisition of metastatic potential, then one can infer that genes involved in controlling particular stages of tumor progression (e.g.,metastasis) are encoded nearby.

A paradigm describing the genetics of metastasis is modeled upon the concepts advanced by Vogelstein and colleagues in colorectal carcinoma. Analogous to the role of oncogenes in tumorigenesis, metastasis-promoting genes drive conversion from nonmetastatic to metastatic. The best characterized is the activated ras oncogene which can confer both tumorigenic and metastatic potential to fibroblasts (reviewed in {Chambers & Tuck 1993 10058 /id}). Analogous to tumor suppressors, metastasis suppressor genes block metastasis. The prototypical metastasis suppressor gene, Nm23, was identified in the murine K1735 melanoma using subtractive hybridization and two human homologs have been identified, both mapping to chromosome 17q {Backer, Mendola, et al. 1993 1806 /id}. Nm23-H1 expression has been correlated inversely with many, but not all late-stage, metastatic human tumors (reviewed in {Freije, MacDonald, et al. 1996 9543 /id}). Likewise, transfection of Nm23-H1 into metastatic human tumor cell lines has resulted in suppression of metastasis, notably MDA-MB-435 {Leone, Flatow, et al. 1993 1132 /id} and the human melanoma cell line, MelJuSo {Miele, De La Rosa, et al. 1997 8530 /id}. The mechanism of action for metastasis suppression by Nm23 still remains unknown {Freije, MacDonald, et al. 1996 9543 /id}.

Subsequently, several other genes have been described as metastasis suppressors (reviewed in {Fidler & Radinsky 1996 7702 /id} {Welch & Goldberg 1997 6584 /id} {Welch & Wei 1998 11672 /id}). Three of them suppress metastasis of human cancer cells in animal models — KAI1, KiSS1 and E-cadherin. KAI1 was discovered in rat prostatic carcinoma cell lines which had been suppressed for metastasis following introduction of human chromosome 11 {Dong, Lamb, et al. 1995 4483 /id}. KAI1 is a glycoprotein belonging to the transmembrane 4 superfamily and is

identical to the CD82 or C33 antigens. The human gene maps to 11p11.2. KAI1 expression generally correlates with breast carcinoma aggressiveness {Yang, Welch, et al. 1997 9573 /id} and, following transfection into MDA-MB-435, suppresses metastasis without affecting tumorigenicity {Phillips, White, et al. 1998 7227 /id}.

KiSS1 was identified using subtractive hybridization comparing metastasis suppressed chromosome 6-human melanoma cell lines with their parents {Lee, Miele, et al. 1997 10007 /id} {Lee, Miele, et al. 1996 7125 /id} {Lee & Welch 1997 7226 /id}. Transfection of KiSS1 into the human melanoma cell lines C8161 {Lee, Miele, et al. 1996 7125 /id} and MelJuSo {Lee & Welch 1997 7226 /id} as well as MDA-MB-435 breast carcinoma cell line {Lee & Welch 1997 8809 /id} resulted in significant suppression of metastasis without inhibiting tumorigenicity. Like Nm23 and KAI1, the mechanism(s) by which KiSS1 suppresses metastasis have not been fully elucidated.

E-cadherin is a cell surface glycoprotein involved in calcium-dependent cell-cell adhesion. Reduced levels of E-cadherin are associated with decreased adhesion and increased grade of epithelial neoplasms {Mareel, Boterberg, et al. 1997 9676 /id}. Mutations of E-cadherin and the associated protein α-catenin have been associated with acquisition of the invasive phenotype {Vermeulen, Bruyneel, et al. 1995 5385 /id} and transfection of E-cadherin decreases motility and invasiveness of cancer cells {Vermeulen, Bruyneel, et al. 1995 5385 /id} {Frixen, Behrens, et al. 1991 7131 /id}. Thus, since E-cadherin decreases release of tumor cells from the primary tumor, it is considered a metastasis suppressor {Perl, Wilgenbus, et al. 1998 11063 /id}. However, there is also evidence that it can function as a tumor suppressor gene {Frixen, Behrens, et al. 1991 7131 /id} {Vermeulen, Bruyneel, et al. 1995 5385 /id} {Christofori & Semb 1999 15140 /id}

The search for breast cancer metastasis genes acknowledges one of the most common changes in late-stage breast carcinoma (familial or sporadic). Losses of genetic material on chromosome 11q occurs in 40-65% of cases. There are several regions spanning the q-arm of chromosome 11 for which associations have been made with breast cancer progression. Among the most common are amplifications and deletions involving and 11q13. Within this region, there is evidence supporting the existence of tumor promoting, tumor suppressing, metastasis promoting and

metastasis suppressing genes in this region. The genes int-2, hst, bcl-1, glutathione S-transferase, CCND1 and EMS-1, which map to 11q13 are amplified in breast cancer at a frequency between 3 to 20%. Introduction of 11q-containing YACs into mouse A9 fibrosarcoma cells suppresses tumorigenicity {Koreth, Bakkenist, et al. 1999 13994 /id}. There exists a high frequency involvement of rat chromosome 1 (which is syntenic to human chromosome 11 {Rinker-Schaeffer, Hawkins, et al. 1994 3668 /id}) in the development and progression of rat mammary tumors {Aldaz, Chen, et al. 1992 1544 /id} {Pearce, Pathak, et al. 1984 86 /id}. Based upon these observations and high frequency deletions involving 11q13-q14 in late-stage, metastatic breast carcinomas {Zambetti & Levine 1993 2249 /id} {Leone, Flatow, et al. 1993 1132 /id} {Van de Vijver 1993 2095 /id}, we tested hypothesis chromosome 11q coated metastasis suppressor gene. Upon finding that introduction of a normal human chromosome 11 into metastatic MDA-MB-435 human breast carcinoma suppressed metastasis without affecting tumorigenicity {Phillips, Welch, et al. 1996 5718 /id}, we set out to identify gene(s) responsible.

DD-RTPCR was used to discover genes more highly expressed in neo11/435 than in their metastatic counterparts. BRMS1, which maps to the region frequently involved in breast carcinoma progression (i.e., 11q13.1-q13.2) was identified and was expressed ≥5-fold more in the metastasis suppressed hybrid clones been in the metastatic parents. When transfected into MDA-MB-435 and MDA-MB-231 cells, both the incidence of metastasis and the number of lung metastases per mouse were significantly inhibited compared to controls. Even when tumor-bearing animals were allowed more time for metastases to develop, metastasis was suppressed. Although tumor development BRMS1-transfected MDA-MB-435 cells was slightly delayed compared to controls, tumors still formed. BRMS1 mRNA was still detectable within the primary tumors (data not shown). Taken together, these data fulfill all requirements that BRMS1 is metastasis suppressor gene. Metastasis is suppressed whereas tumorigenicity is not, even when BRMS1 means expressed within the locally growing tumor.

The mechanism by which BRMS1 suppresses metastasis is still not fully determined; however, several features lead to the hypothesis that this gene encodes a novel protein that functions in a signaling cascade to control transcription. The presence of two nuclear localization domains suggest that BRMS1 localizes to the nucleus. Several potential phosphorylation sites suggest that BRMS1 could be part of a signaling pathway while presence of a coiled-coil periodicity and

imperfect leucine zipper suggest that BRMS1 interacts with other proteins. Collectively, these characteristics suggest that BRMS1 might mediate metastasis suppression by altering the expression of known metastasis-suppressor genes.

To test this possibility, protein levels of KAI1 and Nm23 were measured in BRMS1-transfected MDA-MB-231 cells. KAI1 and Nm23 were both expressed in all of the clones to varying degrees. The level expression of of KAI1 and Nm23 did not appear to correlate with the protein expression of BRMS1 in any of the transfected clones. The similar study was done to measure E-cadherin of levels in the MDA-MB-231 BRMS1-transfected clones. Parental MDA-MB-231 cells did not express E-cadherin nor did any of BRMS1 transfectants. Metastatic MDA-MB-231 cells express high levels of KiSS1 mRNA. Data of the BRMS1-transfected MDA-MB-231 cells exhibited a noticeable change in KiSS1 expression. Taken together, these observations suggest that if BRMS1 is a transcription factor, it is not acting through transcriptional modulation of any of the known human metastasis suppressor genes. Results in these experiments do not, however, preclude the possibility that BRMS1 is interacting directly with metastasis suppresses. One possibility, albeit unproven, is that BRMS1 might interact with Nm23 via the leucine zipper motifs within both proteins {Postel, Berberich, et al. 1993 2094 /id}.

Using a variety of *in vivo* and *in vitro* assays, no simple mechanism emerges. BRMS1 transfectants are still adherent, invasive, and proliferative. The BRMS1-transfected breast carcinoma cells display similar morphology to their metastatic parental cells both *in vitro* and *in vivo*. Curiously, BRMS1-transfected MDA-MB-435 cells acquired acute sensitivity to trypsin treatment. Whether this is related to be decreased in stromal fibrous material present in the tumors remains to be determined. Among the possible mechanisms of action of BRMS1 could be alteration in how the breast carcinoma cells secrete and/or manipulate extracellular matrix. It must be noted, however, that MDA-MB-231 cells did not acquire trypsin hypersensitivity like MDA-MB-435. Other preliminary data that support the role of BRMS1 altering cell-cell and cell-matrix interactions is the acquisition of tight junctional communication in BRMS1-transfected breast carcinoma cells whereas the parental cells exhibited none (M.J. Seraj, M. Saunders, H. Donahue and D.R. Welch, unpublished observations).

BRMS1 is widely expressed in a wide variety of normal human tissues at the mRNA level.

BRMS1 transcript (1.5 kb) was detected in virtually every human tissue examined. The uniform size of BRMS1 transcript suggests that it is not alternatively spliced in various tissues. However because of the presence of relatively broad bands in pancreas and peripheral blood leukocytes, alternative splicing cannot be ruled out . The genomic organization of BRMS1 was not identical in the multiple eukaryotic species examined according to Southern Blot analysis; however, the presence of bands in multiple species suggests that BRMS1 (or related genes) is relatively well conserved (data not shown).

Compared to the many normal tissues examined, BRMS1 mRNA expression was very low in the MDA- MB-435 and MDA-MB-231 cells by both RT-PCR and poly(A)$^+$-enriched mRNA Northern blots. Expression of the protein levels is not yet confirmed because specific antibody/antiserum recognizing native BRMS1 has not yet been developed. A preliminary study was undertaken to measure BRMS1 in a northern blot comprised of a panel of human breast carcinoma cell lines: MCF10A, MCF7, T47D^{CO}, MDA-MB-435, MDA-MB-231, LCC15, SUM185, SUM1315 (http://p53.cancer.med.umich.edu/clines/clines.html) and MKLF {Pauley, Soule, et al. 1993 14590 /id} {Soule, Maloney, et al. 1990 8475 /id} {Wei & Miner 1994 2454 /id} {Sung, Gilles, et al. 1998 11976 /id}. All of the cell lines have characteristics which labeled them as "aggressive" but in our hands only MDA-MB-435 and MDA-MB-231 are reproducibly metastatic in athymic mouse models. Mice the mRNA level, expression was high in LCC15 and MKLF, but expression was observed in MCF10A, T47D, SUM185 and SUM1315 cell lines (data not shown). A more extensive analysis is required. Sequencing is underway to determine whether BRMS1 is wild-type or mutant.

In summary we of found a new human breast carcinoma metastasis suppressor gene by differential display comparing metastatic MDA-MB-435 cells and metastasis suppressed neo11/435 cells. The BRMS1 gene maps to a "hot spot" in breast cancer progression, 11q13, further supporting the likelihood that BRMS1 is important in human breast cancer progression towards metastasis. In general, low expression of BRMS1 correlates with the metastatic potential in human breast carcinoma cells in nude mice. It will be necessary to further analyze BRMS1 gene in other breast carcinomas during various stages of progression. The predicted BRMS1 protein has several features which its role as a transcription factor in a signaling cascade, although dysfunction has yet to be proven. It is intellectually satisfying that any metastasis

suppressor would mediate a variety of downstream pathways. This is consistent with the concept that BRMS1 is a transcription factor. However it must be noted that BRMS1 did not significantly modified the mRNA expression of a variety of known human metastasis suppressor genes. Since the most lethal attribute of breast cancer cells is their ability to spread and colonize distant sites, BRMS1 may represent an early step toward preventing metastasis and improving breast cancer survival.

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Figure legends

Figure 1. Identification of differentially expressed genes in metastasis-suppressed neo11/435 hybrid cell clones using differential display. (**A**) DD-RTPCR result in which *BRMS1* was identified. Replicate reactions were done in parallel to compare parental, metastatic MDA-MB-435 (Lanes 1 and 2) with metastasis-suppressed mixtures (1:1:1) of neo11/435 clones A3, B1 and D1 (Lanes 3 and 4). Lanes 2 and 4 contained twice as much starting material as Lanes 1 and 3. An equal mixture of neo11/435 hybrid cell clones was used to minimize the impact of tumor heterogeneity on the differential display reaction. (**B**) Northern blot analyses using candidate differential display products as probes. Only candidates displaying ≥5-fold higher expression in neo11/435 hybrid cell clones are presented. Poly(A)+ mRNA (2 μg) was electrophoresed on denatured agarose gels, transferred to a nylon membrane and fixed and probed with random-prime radiolabeled PCR products from the differential display reaction. Equal loading was verified by probing with GAPDH cDNA. Approximate transcript sizes are depicted to the right of each gel. The gels labeled 1, A1 and G1B5 represent the known genes (N-acetylgalactosamine-6-sulfate-sulphatase, adenine phosphoribosyltransferase and hexokinase II) identified in the differential display reactions. (**C**) Differential expression of the novel genes was quantified using phosphor image analysis. Relative expression was compared to parental MDA-MB-435; and only genes showing ≥5-fold higher (reference line) expression in the neo11/435 hybrid cell clones were chosen for further study. (**D**) Expression of BRMS1 mRNA in normal tissues. Multi-tissue RNA blots [2 μg of poly(A)+ mRNA per lane] were purchased from Clontech Laboratories Inc..The blot was hybridized using full-length *BRMS1* probe as described above. Detection of *BRMS1* message (1.5 KB) was possible following overnight exposure. Alternative splicing was not evident in any of these lanes. Lanes are loaded in the following order: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testes, ovary, small intestine, colon, and peripheral blood leukocytes. To separate gels were used for these experiments.

Figure 2. Nucleotide and predicted amino acid sequence of *BRMS1*.

Figure 3. Northern blot (Panel A) of MDA-MB-435 and MDA-MB-231 parental (P) and *BRMS1*-transfected cell clones. GAPDH was used to verify equal loading and transfer. Panel B: Western blot (50 μg/lane) measuring expression of BRMS1-901 (epitope-tagged) in MDA-MB-231 parental (P), pcDNA3 vector (V) and *BRMS1*-transfectants. The clones in panel A (right six lanes) are the same ones evaluated in panel B. Note: relative mRNA and protein expression are similar for BRMS1 transfectants. The blots were stripped and re-probed with antibodies/antisera to the human metastasis suppressor genes KAI1 and Nm23, with β-tubulin and β-actin used to monitor equal loading. Protein loading for *BRMS1*-transfectant

clones 3, 4, and 5 was lower than the other lanes. This was most evident with regard to the β-tubulin detection. A similar experiment was done to measure expression of E-cadherin using the same clones. E-cadherin positive control (+) was included. Parental MDA-MB-231 and none of the clones expressed E-cadherin. Expression of the other metastasis suppressors did not correlate with BRMS1 expression.

Figure 4. BRMS1-transfected MDA-MB-231 and MDA-MB-435 cell morphology is similar *in vitro* (Panel A, Magnification = 100X). BRMS1-transfected MDA-MB-435 tumor histologic appearance is similar to that of parental, metastatic MDA-MB-435 (Panel B, Magnification = 160X). Cells (1×10^6) were injected into the mammary fat pad of athymic mice. Tumors were removed approximately 4 weeks after injection, fixed in formalin, sectioned and stained with hematoxylin and eosin. The tumor formed by MDA-MB-435 cells exhibited tightly packed cells with pleomorphic nuclei and prominent nucleoli. Fibrous bands (arrows) are present throughout the tumor. Tumors formed by the BRMS1-transfected cells were histologically similar, with tightly packed cells, nuclear degeneration and occasional mitotic figures (arrowhead). The stromal elements of these two tumors, however, were somewhat distinct (i.e., BRMS1-transfectant tumors lacked the fibrous bands). Growth of BRMS1-transfected MDA-MB-435 tumor cells following injection into the mammary fat pad was modestly delayed (Panel C) whether BRMS1 was native or epitope-tagged (*).

Figure 5. BRMS1-transfected MDA-MB-231 cell clones are significantly ($P<0.05$) suppressed for formation of pulmonary metastases following intravenous injection into the lateral tail veins of athymic mice compared to control cells. Parental (P) and pcDNA3 vector-only transfectant (V) are controls for two independent experiments. An uncloned population of BRMS1-transfected MDA-MB-231 (mix) is also included in these experiments. Metastasis suppression generally correlated with the level of BRMS1 mRNA and protein expression (see Figure 3).

Table 1. Differentially expressed cDNAs isolated using DD-RTPCR of metastasis-suppressed neo11/MDA-MB-435 hybrid cell clones compared to metastatic MDA-MB-435

Fragment	Size (bp)	Homology	% homology
BRMS1 [a]	634	novel	–
F5A3	174	novel	–
8A3	454	novel	–
1	370	adenine phosphoribosyltransferase	99 [b]
A1	387	N-acetyl-galactosamine-6-sulfate sulfatase	100
G1B5	673	hexokinase II	97

[a] BRMS1 – breast cancer metastasis-suppressor gene 1. F5A3 and 8A3 are interim nomenclature only. They will be renamed *BRMS2 and BRMS3*, respectively, following confirmation of function.

[b] Note: Although the levels of homology between the PCR products and the known proteins are high, the expected size on Northern blotting did not corroborate the identity of the known genes (APRT ≈ 0.8 kb; GALNS ≈ 1.6 kb; HKII ≈ 5.3 kb). No further follow-up for genes with high homology was undertaken.

Table 2. BRMS1 suppresses metastasis of MDA-MB-435

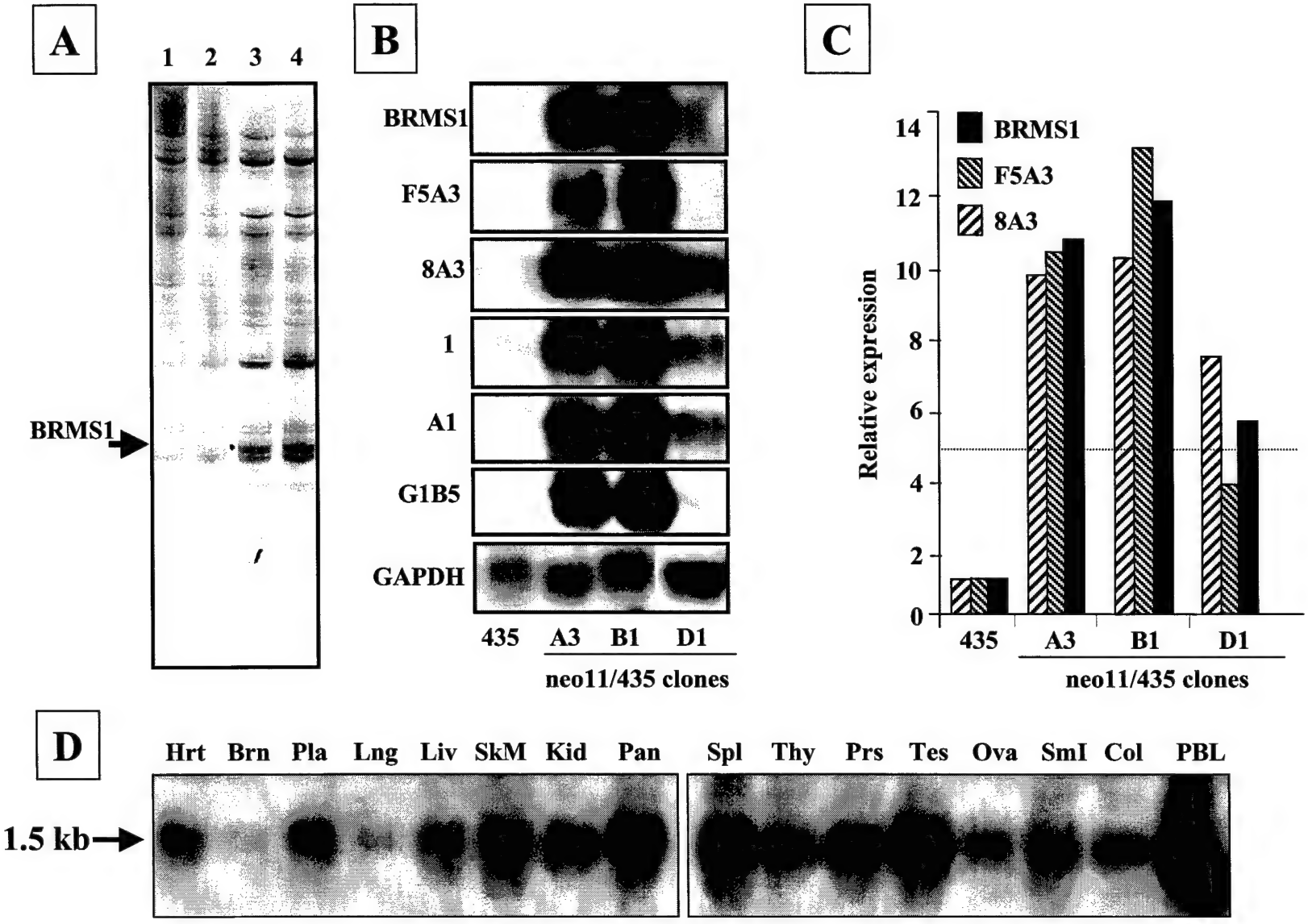
Cell line	Incidence of metastases			
	Lung		Extrapulmonary	
	(No. mice with metastases/No. mice injected)	P<0.05	(No. mice with metastases/No. mice injected)	P<0.05
MDA-MB-435	9/15		15/15	
MDA-MB-435/pcDNA3	9/13		13/13	
BRMS1.3 (901-tagged)	2/15	***	3/15	***
BRMS1.4 (901-tagged)	5/14		5/14	
BRMS1.6 (901-tagged)	2/15	***	0/15	***
BRMS1.1	2/8	***	0/8	***
BRMS1.3	1/8	***	3/8	***
BRMS1.5	3/7		5/8	

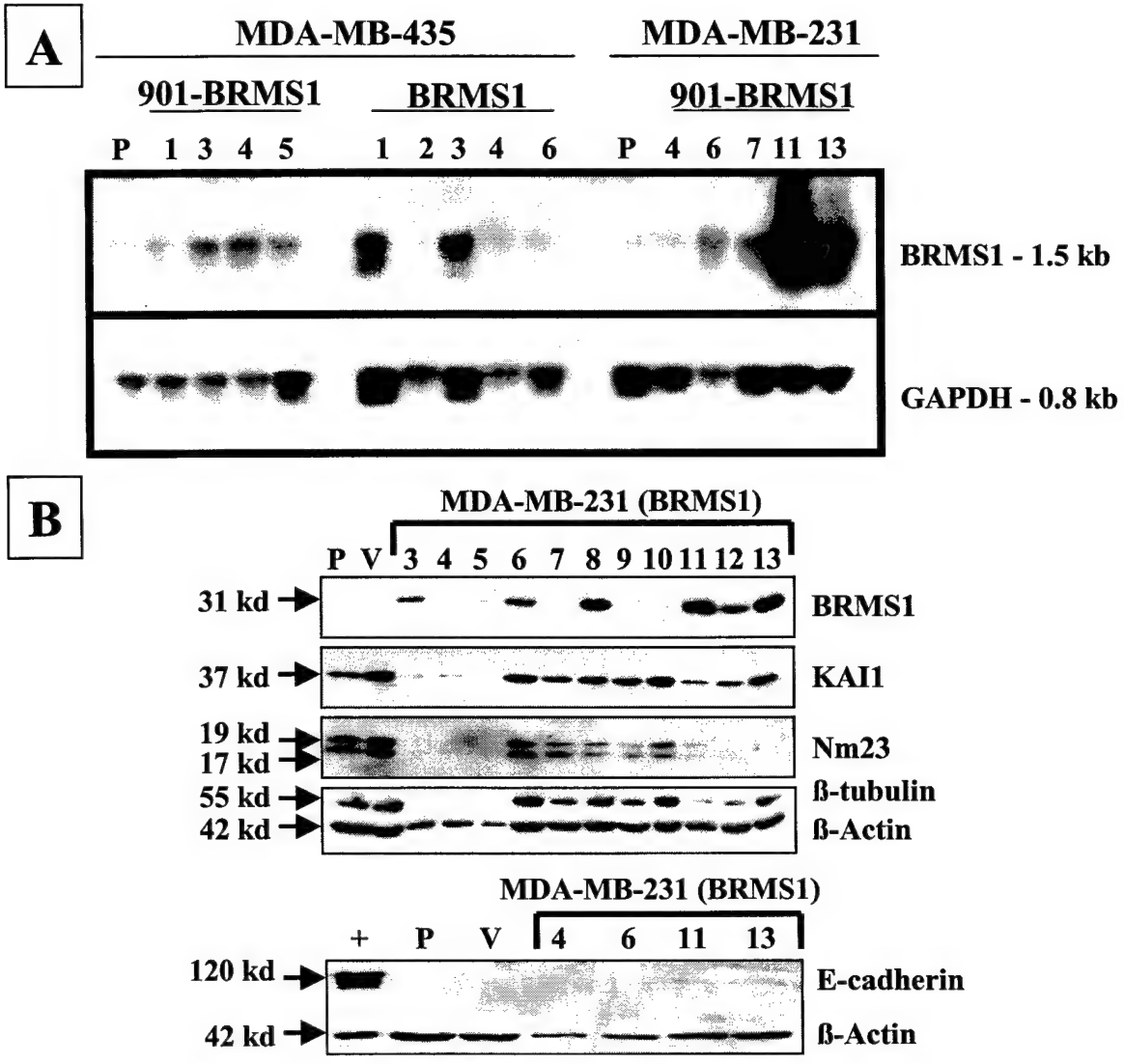
Cells (1 x 10^6) were injected into the axillary mammary pads of 5-6 wk old female athymic mice. Tumors were removed when the mean tumor diameter reached 1.3-1.5 cm. Four weeks later, mice were killed and presence of metastases determined.

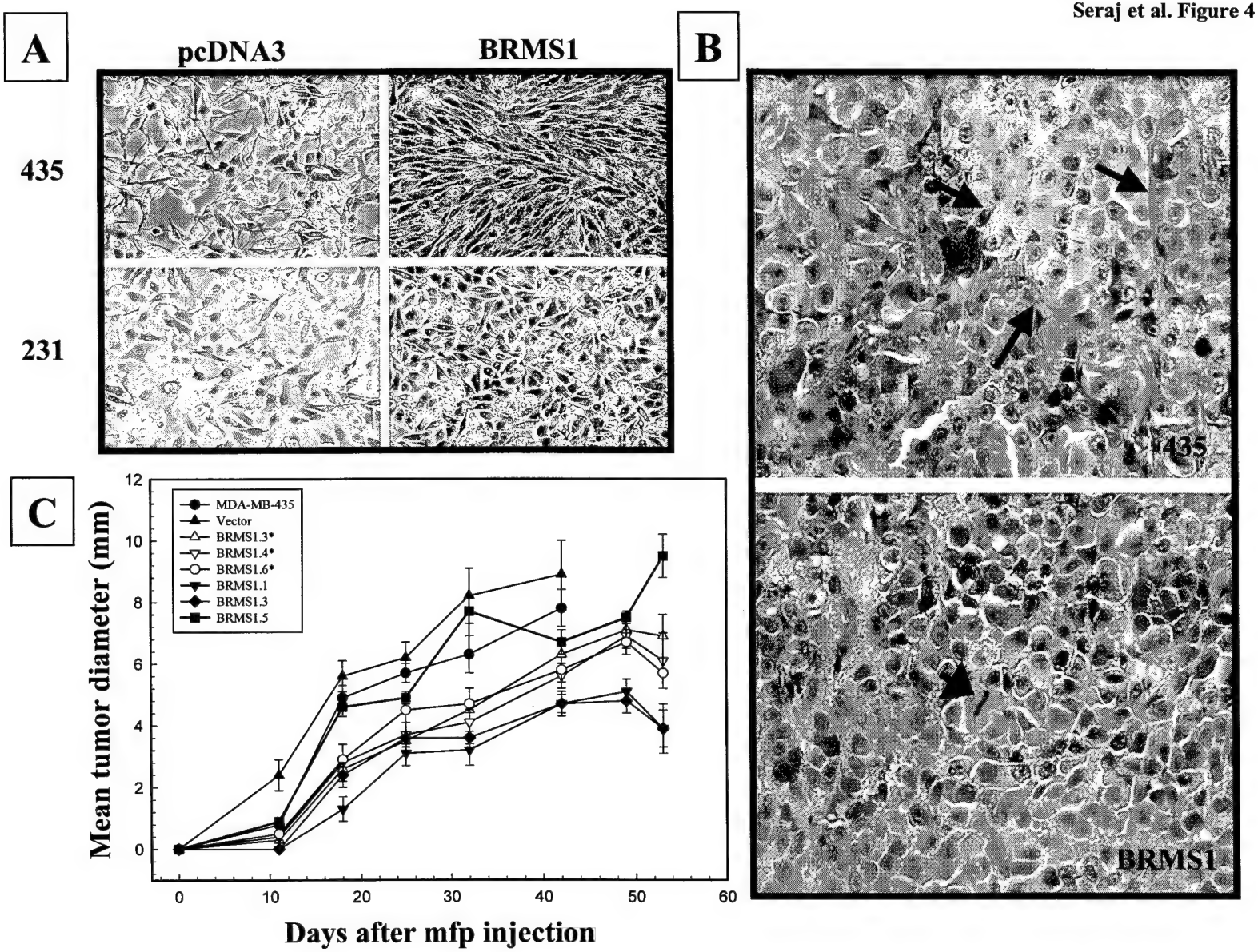
The number and incidence of lung metastases were compared to vector-only transfected MDA-MB-435 using the Kruskal-Wallis ANOVA followed by Dunn's post-test. Incidence of extrapulmonary metastases (usually ipsilateral axillary lymph nodes, but occasionally ribcage, diaphragm and chest wall) was similarly examined.

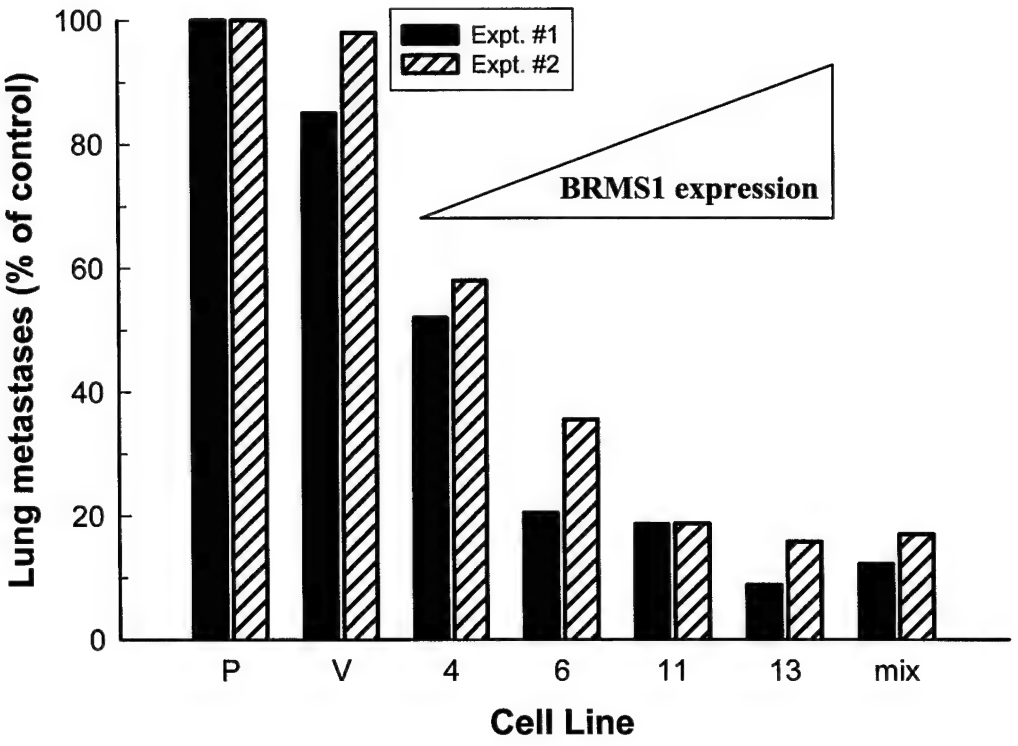
Data are pooled from two independent experiments involving 7-8 mice per group. Data for the native BRMS1 (i.e., not epitope-tagged) was collected from a single study.

References


A
1 2 3 4
BRMS1
B
BRMS1
F5A3
8A3
1
A1
G1B5
GAPDH
435 A3 B1 D1
neo11/435 clones
C
BRMS1
F5A3
8A3
Relative expression
0 2 4 6 8 10 12 14
435 A3 B1 D1
neo11/435 clones
D
Hrt Brn Pla Lng Liv SkM Kid Pan Spl Thy Prs Tes Ova SmI Col PBL
1.5 kb


A
MDA-MB-435
MDA-MB-231
901-BRMS1
BRMS1
901-BRMS1
P 1 3 4 5 1 2 3 4 6 P 4 6 7 11 13
BRMS1 - 1.5 kb
GAPDH - 0.8 kb
B
MDA-MB-231 (BRMS1)
P V 3 4 5 6 7 8 9 10 11 12 13
31 kd
BRMS1
37 kd
KAI1
19 kd
17 kd
Nm23
55 kd
ß-tubulin
42 kd
ß-Actin
MDA-MB-231 (BRMS1)
+ P V 4 6 11 13
120 kd
E-cadherin
42 kd
ß-Actin


A
pcDNA3
BRMS1
435
231
B
435
BRMS1
C
MDA-MB-435
Vector
BRMS1.3*
BRMS1.4*
BRMS1.6*
BRMS1.1
BRMS1.3
BRMS1.5
Mean tumor diameter (mm)
Days after mfp injection
0
2
4
6
8
10
12
0
10
20
30
40
50
60


Lung metastases (% of control)
100
80
60
40
20
0
Expt. #1
Expt. #2
BRMS1 expression
P
V
4
6
11
13
mix
Cell Line

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Identifying and characterizing metastasis-suppressor genes in human cancer

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Running title: KiSS1 metastasis suppressor

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Abbreviations: MMCT, microcell-mediated chromosomal transfer;

The most lethal attribute of cancer cells is their ability to establish secondary colonies at distant sites. Metastasis is the culmination of tumor cell evolution toward increasing autonomy. Fortunately, only a small subset of tumor cells acquire this complex phenotype. Most neoplasms arise from single mutated cells but are inherently unstable and become heterogeneous as they accumulate genetic alterations [1; 2]. Metastatic cells acquire requisite genetic and phenotypic changes by intrinsic genetic means upon which extrinsic selection pressures are applied [3]. While the exact mechanisms leading to malignancy are poorly understood, it is clear that metastatic cells must have the ability to complete a complex series of steps in order to form secondary foci: growth of the primary tumor, neo-vascularization, invasion into surrounding tissue, intravasation into the blood vessels and/or the lymphatic system, circulation and dissemination, attachment to endothelial cells or basement membrane at the secondary site, extravasation, migration and finally, growth at the target organ (reviewed in [4-11]). It is now believed that this process is controlled by a finite number of key genes [10-14].

Genes that control metastasis may be broadly placed into two categories: those which inhibit metastasis ("metastasis-suppressors") and those which drive metastasis formation ("metastasis promoters") [10; 11; 13]. These categories are the late-stage equivalents to tumor suppressor genes and oncogenes. However, they are distinct in one key way — all tumor suppressor genes are, by definition, also metastasis suppressors since any cell that cannot form a tumor cannot metastasize. However, metastasis-suppressor genes are those for which expression results in a non-metastatic phenotype, but which leave tumorigenicity unaffected. Therefore, metastasis suppressor genes control processes subsequent to tumor formation, demonstrating specificity.

The location of metastasis-controlling genes can be inferred from clinical cytogenetic data. Some chromosomal aberrations are shared between tumor types, while the location of other chromosomal differences are relatively specific. For malignant melanoma, among the most common chromosomal aberrations are deletions involving the long arm of chromosome 6. Similarly, deletions involving chromosome 11 are often seen in late-stage breast carcinomas. Using microcell-mediated chromosomal transfer, introduction of chromosome 6 into metastatic human melanoma cell lines [15; 16] or chromosome 11 into a metastatic human breast carcinoma cell line [17] suppressed metastasis in athymic mouse models. Tumorigenicity was not affected in any of these cases. Conversely, chromosome 11 had no affect on the tumorigenicity of melanoma cell line C8161 and chromosome 6 did not affect metastasis of MDA-MB-435 breast carcinoma cells.

The observations described above demonstrate several important points. First, genes controlling metastasis are distinct from those involved in tumor formation. Second, tumorigenic cells acquire metastatic capability only after accumulating additional genetic defects. Third, cells may not necessarily share the same regulatory pathways for the metastatic phenotype. Collectively, our results imply that mutations of genes on chromosome 6, which are late events in melanoma progression, may be responsible for metastatic potential, and that metastasis-suppressor gene(s) may be encoded on chromosome 6 or be regulated by genes encoded on that chromosome. The obvious question is: what gene(s)? Similar logic can be used for chromosome 11 in breast carcinoma.

To identify the gene(s) responsible, modified differential display and subtractive hybridization approaches were initiated. Candidate metastasis-suppressor genes have been identified and data will be presented for both melanoma and breast carcinoma.

Melanoma metastasis-suppressor genes: Comparison of metastasis-suppressed human melanoma hybrids (neo6/C8161) with matched parental populations (C8161) by subtractive hybridization was utilized to identify several candidate genes [18-20]. The best characterized of these candidates is KiSS1. KiSS1 exhibits extremely low or undetectable mRNA expression in metastatic melanomas, but can readily be detected in normal melanocytes and in cell lines derived from a radial growth phase (benign) melanoma [20]. Unfortunately, determination of protein expression patterns is not yet possible since antibodies recognizing mammalian KiSS1 are not yet available.

Transfection of full-length KiSS1 under the control of a strong, constitutive promoter (CMV) into multiple human cancer cell lines (melanoma and breast) suppressed metastasis without affecting tumorigenicity [10; 18; 19]. Thus, KiSS1 satisfies the criteria of a metastasis-suppressor gene (i.e., metastasis is suppressed, but tumorigenicity is not).

The mechanism of action of KiSS1 remains enigmatic. Thus far, data only point to what KiSS1 does not do. Metastasis-suppressed, chromosome 6 hybrids and KiSS1 transfectants grow slightly more slowly than their parental counterparts, but slower growth was eliminated as key since tumor cells injected into mice were allowed compensatory time to grow. Still, metastases did not develop. Metastasis-suppressed hybrids and KiSS1 transfectants were equally invasive as assessed *in vivo* as well as *in vitro* [15; 16; 21]. Tumor cells have even been detected in efferent vessels [15; 16], suggesting that the suppression of metastasis is downstream of this step in the metastatic cascade.

Just as invasiveness does not appear different, adhesion to endothelial monolayers or extracellular matrix components is not significantly different in neo6/melanoma and KiSS1-transfectant cells. Metastasis suppression does not appear due to increased susceptibility to NK cell killing since metastases were not observed in athymic/beige mice (M.E. Miele and D.R. Welch, unpublished observations). Similarly differences in sensitivity to humoral immune mechanisms are unlikely since the same metastasis suppression was also seen in severe combined immunodeficiency mice (SCID, *xid*) (S.F. Goldberg and D.R. Welch, unpublished observations). Finally, metastasis inhibition does not appear due to failure to induce angiogenesis since vessel counts are not grossly different (R. Radinsky and D.R. Welch, unpublished preliminary results).

A modest reduction of pseudopod extension was found in the neo6/C8161 hybrids compared to the metastatic C8161 cells, suggesting that metastasis suppression by the exogenous chromosome 6 may be the result of alterations in cell motility [21]. These observations were made using collagen type IV as a chemoattractant. Coupled with the predicted protein sequence information (see below), we speculate that metastasis suppression due to chromosome 6 introduction is due, in part, to altered "outside-in" signaling of the chemoattractant.

Restoration of KiSS1 expression correlated with a reduced ability to form colonies in both soft

(0.3%) or hard (0.9%) agar [20]. Clonogenicity in hard agar has been associated with metastatic propensity [22], but there are exceptions to this correlation. Nonetheless, this data implies that KiSS1 may be involved in cell growth (although growth on plastic substrates is unaffected) or cell shape. These observations have been further extended in a collaboration with Drs. Manuel Rieber and Mary Strasberg-Rieber. Briefly, metastatic melanoma cells have the ability to proliferate on suboptimal adhesion matrices (i.e., bacterial petri dishes), a condition under which normal melanocytes undergo apoptosis. Interestingly, neo6/C8161 cells and KiSS1 transfectant clones display an intermediate phenotype. That is, they undergo growth arrest, apparently entering a quiescent phase. We do not yet know how long the cells can remain in this state. This is accompanied by a corresponding shift in the expression of cell cycle and apoptosis genes (M. Rieber, M.S. Rieber, and D.R. Welch, manuscript submitted). These data suggest that metastatic cells can thrive under conditions which favor apoptosis for normal cells. While the nonmetastatic cells do not thrive under these conditions, they can apparently tolerate it. This finding has implications not only for the function of melanoma metastasis suppression, but perhaps for understanding occult disease as well.

Taking all of these results together, our working hypothesis is that KiSS1 controls metastasis at a terminal step in the metastatic cascade (i.e., after arrest in the microvasculature). Preliminary data support this contention. Metastatic C8161 cells and nonmetastatic neo6/C8161 cells have been engineered to produce Enhanced Green Fluorescent Protein (GFP). Kinetic studies indicate that the nonmetastatic cells arrest in the lung, but do not proliferate (S.F. Goldberg, J.F. Harms and D.R. Welch, manuscript in preparation). Whether the occult cells are viable and capable of eventually forming metastases is not known. Certainly, previous experiments in which several months elapsed following injection without development of macroscopic metastasis, would argue that this is not the case. However, additional studies are underway to explore this possibility.

Clues into KiSS1 function can be gained by comparing nucleotide and amino acid sequences to known genes/proteins in the databases. KiSS1 encodes a novel gene that encodes a mostly hydrophilic protein of 145 amino acids ($M_r \approx 15.4$ kDa) which contains several consensus phosphorylation site motifs. Based upon the sequence information alone, we hypothesized that KiSS1 is a cytoplasmic signaling molecule. We have initiated three parallel strategies for assessing KiSS1 mechanism of action. Generation of site-directed and truncation mutations is underway, but the data are too preliminary to report. Subcellular localization studies utilizing chimeric KiSS1●GFP suggest that KiSS1 is indeed located in a cytoplasmic compartment (J.F. Harms, S.F. Goldberg and D.R. Welch, manuscript in preparation). More detailed studies are underway to determine which compartment(s). Finally, yeast two hybrid experiments using KiSS1 as "bait" have been used to identify interacting proteins. Among the candidate interactors identified in this screen was the θ isoform of 14-3-3, a ubiquitous protein implicated in various signal transduction pathways. 14-3-3 family members have been associated with the formation of protein complexes, and are known to bind phosphoserine residues [23-25]. Other genes identified encode 1) a protein containing a tetratricopeptide repeat domain, which is likewise associated with the formation of protein complexes and shares significant structural homology with the binding region of 14-3-3 [26], and 2) a human HLA-B associated transcript [27]. Several novel putative

interactors have also been identified (S.F. Goldberg, J.E. Hopper and D.R. Welch, unpublished observations). Collectively, these data are consistent with KiSS1 functioning in signal transduction.

Surprisingly, the KiSS1 gene is not encoded on chromosome 6. Rather it maps to chromosome 1q32, suggesting that the gene is regulated by genes on chromosome 6. Moreover, the data strongly suggest that intact KiSS1 was present and functional in the metastatic C8161 and MelJuSo cell lines, but is not expressed. From this, one can infer that the defect in these cells is loss of a KiSS1 transcriptional regulator. The identity of the regulator is still unknown although genomic sequence has recently been identified [28] (R.S. Samant, unpublished observations). However, some clues exist. KiSS1 mRNA is undetectable in a highly metastatic C8161 cell line into which an exogenous human chromosome 6 with a complex deletion involving 6q16.3-q23 has been introduced [19]. This result suggests that genes encoded within 6q16.3-q23 are involved in regulation of KiSS1 expression.

Expression of KiSS1 has been evaluated in various normal human tissues by RNA blotting. Abundant KiSS1 transcript was found in the placenta, with weak expression evident in the kidney (detectable only after long exposure) and pancreas. The transcript sizes detected in pancreas (0.8 kb) and kidney (0.9kb) were different than that in placenta (1.0 kb) suggesting the possibility of tissue-specific alternative splicing or processing. Probing a human RNA master blot (Clontech, Palo Alto, CA) which contained RNA samples from 50 different human tissues showed a similar expression pattern, with additional weak hybridization shown for brain (whole, hippocampus, occipital lobe, substantia nigra), and fetal thymus, kidney, spleen, lung, and liver (Md. J. Seraj and D.R. Welch, unpublished observations). A zoo blot was probed with full-length KiSS1 cDNA to assess whether the KiSS1 gene (or family members) are present in other species. The probe hybridized to distinct DNA bands in several species, including cow, dog, mouse, rat, and chicken (S.F. Goldberg and D.R. Welch, unpublished observations). This suggests that KiSS1 is evolutionarily conserved and may even suggest that it is involved in controlling metastasis in other species.

KiSS1 was not the only candidate metastasis suppressor found in the subtractive hybridization experiments. Seven candidate metastasis-suppressor cDNAs were identified with quantitatively (our arbitrary cut-off was $\geq$10-fold) or qualitatively higher levels of expression in the non-metastatic variants. Four of the sequences represented known genes, including HMG-1(Y), AP-2A, and a novel isoform of Nucleophosmin B23. All of these genes are involved in transcriptional regulation. The forth gene, 16A7, is a partially sequenced cDNA fragment of unknown function [20]. Three of the identified cDNA clones, including KiSS1 had no significant homology to known genes.

Although clinical correlations have not been established for KiSS1 in human cancer specimens, the biological data are intriguing and promising. For melanoma, in particular, the need for more accurate and less subjective markers to discriminate benign from malignant lesions (i.e., radial growth phase from vertical growth phase) is essential. Recent studies have shown a high level of discordance among the preeminent dermatopathologists, demonstrating clearly how difficult this problem is [29-31].

Breast carcinoma metastasis-suppressor genes

The genetic underpinnings of breast cancer development and progression are even more complex in that they are in melanoma. Nonetheless, some patterns of karyotypic abnormality accompanying acquisition of malignant characteristics have emerged. Among them, loss of heterozygosity (LOH) involving the long and short arms of chromosome 11 has been observed frequently in late-stage breast carcinomas (reviewed in [11]). To determine whether chromosome 11 harbors a human breast carcinoma metastasis-suppressor gene, studies analogous to those performed in melanoma were initiated.

Microcell-mediated chromosome transfer of chromosome 11 into the metastatic human breast carcinoma cell line MDA-MB-435 resulted in greater than 95% suppression of metastasis [32]. It is important to note that all of these studies were done following injection of MDA-MB-435 into the mammary fat pad. This cell line infrequently metastasizes following intravenous injection [33]. As in the melanoma experiments, tumorigenicity was not inhibited in the neo11/435 hybrids.

Since LOH had been described for loci at 11p15 in breast carcinoma [34-36] and since the KAI1 prostate metastasis-suppressor gene mapped to approximately the same region [37; 38], we tested whether KAI1 might be the metastasis suppressor implicated in the microcell transfer experiments. We also asked whether the KAI1-related gene, TAPA1, mapping to the same region might be responsible [17].

Both KAI1 and TAPA1 protein expression were evaluated by Western blotting and found to be more highly expressed in the neo11/435 hybrid cell lines. However, transfectants of MDA-MB-435 having full-length KAI1 cDNA did not exhibit decreased metastatic potential. Interpretation was complicated since the metastases expressed lower KAI1 than the primary tumor or the corresponding cells in culture. Moreover, the KAI1 protein appeared to be differently glycosylated in the transfectants than in the neo11/435 hybrids. Thus, using functional data as a criterion, it is still not certain whether KAI1 is a *bona fide* human breast carcinoma metastasis suppressor gene. However, KAI1 expression levels did appear to correlate with breast tumor progression at the mRNA level [39].

A modified differential display approach was undertaken to identify metastasis suppressor genes in neo11/435 hybrids. This series of experiments was more difficult than the melanoma studies for the following reasons. First, the metastasis suppression was not complete. As a result, the impact of heterogeneity on differential gene expression complicated the differential display. To compensate equal mixtures of mRNA from four neo11/435 clones were compared to parental MDA-MB-435. Since the latter was not clonal, the impact of "noise" was decreased.

Six candidate genes which were expressed ≥ 5 fold more in neo11/435 hybrids versus metastatic, parental MDA-MB-435 cells were identified [40]. Three of the cDNAs represented novel genes (8A3, G1A2, F5A3) while the other three cDNAs were homologous to known genes (N-acetylgalactosamine 6-sulphatase, adenine phosphoribosyltransferase and hexokinase II). Full-length cDNAs of the novel genes have been isolated, introduced into a mammalian expression vector. Experiments to directly test their effects on metastatic potential are underway.

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Laboratory Techniques in Biochemistry and Molecular Biology
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In vivo cancer metastasis assays

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Metastasis is defined as the formation of tumors that are discontinuous from the primary tumor. These secondary tumors can be at nearby or distant sites and can form following dissemination of cells via lymphatic, hematogenous, coelomic cavities or epithelial cavities (Willis 1973). The most common routes for metastatic spread are lymphatic and hematogenous metastasis; so, secondary tumor formation via those routes will be the focus in this chapter. However, it must be noted that these routes are not necessarily the common ones for spread of some tumor types (e.g., ovary (Cannistra 1993)).

For a cell to successfully colonize a secondary site, it must complete every step of a complex, multistep cascade (Fidler & Radinsky 1990; Radinsky & Fidler 1992). Malignant cells invade adjacent tissues and penetrate into the lymphatic and/or circulatory systems. Then tumor cells detach from the primary tumor and disseminate. During transport, cells travel individually or as emboli composed of tumor cells (homotypic) or tumor cells and host cells (heterotypic). At a secondary site, cells or emboli either arrest because of physical limitations (e.g., too large to traverse a capillary lumen) or by binding to specific molecules in particular organs or tissues. Once there, tumor cells then proliferate either in the vasculature or extravasate into surrounding tissue (Chambers, *et al.* 1995; Koop, *et al.* 1996; Luzzi, *et al.* 1998). To form macroscopic metastases, cells must then recruit a vascular supply (Ellis & Fidler 1995; Folkman 1995; Kohn & Liotta 1995; Rak & Kerbel 1996; Weinstat-Saslow & Steeg 1994; Weinstat-Saslow, *et al.* 1994) and respond appropriately to the tissue's environmental milieu by proliferating (Nicolson 1994; Radinsky 1995a; Radinsky 1995b). Less than 0.1% of cells that intravasate survive to form clinically detectable, macroscopic metastases (Fidler 1970; Tarin, *et al.* 1984). At which step(s) of the metastatic cascade circulating tumor cells commonly succumb is debatable (Chambers, *et al.* 1995; Koop, *et al.* 1995; Koop, *et al.* 1996) .

The very nature of the metastatic process dictates that the organization of this chapter differ from other chapters dealing with *in vitro* studies. At the outset, it is important to acknowledge that there are as many variations for how to study metastasis *in vivo* as there are people doing them. While the chapter will conclude with specific technical recommendations, most of the chapter will outline the theoretical considerations and rationale for experimental design and interpretation of metastasis assays.

Why study metastasis in vivo?

Metastasis is not equivalent to invasion, adhesion, growth rate, susceptibility to immune cell killing, or any of the many steps in the metastatic cascade. These processes are necessary for successful colonization of secondary sites, but they are not sufficient for a cell to be metastatic. Failure to distinguish between individual steps from the complete process of metastasis has contributed greatly to confusion and misinterpretation in the scientific literature. Just because a cell line is highly invasive or adheres strongly to extracellular matrices does not necessarily translate to its having the ability to metastasize. Yet, this type of faulty extrapolation is common.

Despite the wealth of useful information than can be gleaned from *in vitro* assays measuring a step(s) in the metastatic cascade, the only method for assessing metastatic potential involves the use of *in vivo* models. *In vitro* models are simply not of sufficient complexity to recapitulate the

multitude of steps of the metastatic process. This is not to demean the usefulness of *in vitro* models. To the contrary, they are extremely useful because *in vitro* studies minimize or eliminate variables that complicate interpretation, contribute to inter-experimental variability, and cloud model development. Other chapters in this volume will focus on the proper use of and interpretation of *in vitro* assays of adhesion, invasion, etc. The focus of this chapter will be on the methodologies used to study metastasis *in vivo*. Parallel studies utilizing both *in vivo* and *in vitro* approaches are powerful strategies for dissecting the biochemical and molecular basis for cancer metastasis.

Another common issue relates to the relationship between tumorigenicity and metastasis. They are distinct phenotypes. Tumorigenicity is a prerequisite to metastasis, but metastasis is a property of only a small portion of cells within a neoplasm (reviewed in (Chambers, *et al.* 1995; Fidler & Nicolson 1987; Luzzi, *et al.* 1998; Weiss 1990)). Metastatic cells represent a subpopulation having additional capabilities to those required for uncontrolled growth. So, in order to study metastasis, models designed specifically for this purpose are necessary. Moreover, the appropriate use of these models is required.

What defines an appropriate model of metastasis?

As long as a model allows testing of a defined hypothesis, its use is appropriate. However, the quality of the model for emulating the pathobiology of human disease determines whether the model is relevant. Relevance is difficult to define as all models are inadequate for studying some aspects of metastasis. It is beyond the scope of this chapter to evaluate the validity of individual models. Readers are referred to other reviews which more adequately cover this area (Welch, *et al.* 1986; Welch 1986; Welch 1997).

Two criteria must be met if a model is to be considered useful for studying metastasis. First, one must use metastatic cells. Unfortunately, it is still commonplace that many studies utilize nonmetastatic cells for studies of metastasis. The primary reason for this is the rationalization that if cells are derived from metastases, they are *de facto* metastatic. This conclusion does not necessarily follow. For example, virtually all human breast carcinoma cell lines were isolated from metastases or pleural effusions; however, few (e.g., MDA-MB-435 and MDA-MB-231) reproducibly form macroscopic metastases in a majority of immunocompromised mice (Sung, *et al.* 1998) . Additionally, some investigators started their studies with metastatic cells, but the conditions under which the cells were maintained rendered them nonmetastatic. This does not necessarily mean that cell culture maintenance was flawed. Rather it could simply reflect the inherent phenotypic instability of tumor cells (Cheng & Loeb 1993; Nowell 1976). Nonetheless, phenotypic instability and inconsistent culture conditions have resulted in several variants of cells with the same name, but profoundly different biologic properties. Therefore, it is incumbent upon every investigator to replicate previously published experiments in his/her laboratory prior to initiating further studies. This point cannot be overemphasized.

The second criterion is that tumor cells must be compatible with the animal model. This is analogous to Paget's "Seed and Soil" hypothesis (Paget 1889). This parameter may explain the difficulties in obtaining relevant models for certain tumor types and/or sites of metastatic

colonization.

Cell lines

The most critical component for the study of metastasis is metastasizing cells. Since most people obtain their cells from cell culture, it is important to outline some of the criteria for the proper preparation and maintenance of those cultures. Details are omitted regarding verification of species of origin (karyotype, isozyme expression, etc.), tissue of origin (surface markers, enzyme expression patterns) and absence of opportunistic infections. It is presumed that such characterization will be done by readers of this volume before any studies are undertaken. Another assumption is that the conditions used to culture the cells have been optimized. That is, the cells are not allowed to undergo stress due to neglect. If this happens, unintended selection will have occurred and stability of cellular behavior cannot be ensured. The third assumption is that the culture conditions used are standardized. That is, cells will be cultured under identical conditions (e.g., same medium, culture plates, serum type and concentration, detachment procedures, etc.) to those utilized in previously published papers. Although the latter is intellectually apparent, it is least the one least adhered to by investigators new to the metastasis field. The following section outlines the reasons why this parameter deserves focused attention of an investigator.

Cells should never be used unless they are at least 90% viable. Dead cells can modify the behavior of viable cells. Fidler, using B16 melanoma cells, showed that presence of lethally irradiated cells within an inoculum greatly changed metastatic potential (Fidler 1973). This situation is reminiscent of the so-called feeder layer or Revesz effect seen in survival curve data (Puck, *et al.* 1956; Revesz 1958; Revesz 1956). Yet the mechanisms are not known.

Even when culture conditions are optimized and great care is taken to minimize iatrogenic effects, tumor cell populations display greater genetic instability than their normal counterparts. Metastatic cells are no exception. At diagnosis, tumors are already complex mixtures of cells despite the fact that the vast majority of tumors are clonal in origin. Among the earliest detectable changes in transformed cells (anchorage-independent growth, not contact-inhibited, and immortal) is genetic instability (reviewed in (Greller, *et al.* 1996; Heppner & Miller 1997; Tlsty 1997)). Even before they become tumorigenic, transformed cells display genomic instability that is apparently the driving force for further progression. Genomic plasticity is crucial for the generation of intratumoral heterogeneity (Cheng & Loeb 1993; Tlsty 1997; Welch & Tomasovic 1985). Heterogenous populations are subjected to selection pressures that drive evolution toward increasingly malignant characteristics (i.e., invasion and metastasis). Besides the inherent differences between cells at the genetic level, cells also respond to signals from the host and other tumor cells. Thus, it is common to observe phenotypic drift over time (reviewed in (Nowell 1976; Welch & Tomasovic 1985)). This change is often gradual, presumably as proportions of different clones change within the population, but the rate and direction of drift are clone-dependent. To minimize the impact of phenotypic drift (since there is currently no know way to eliminate it), behavior must be periodically compared to a baseline. When behaviors change (i.e., when metastatic potential increases or decreases, or when distribution of

metastasis is altered), frozen aliquots from a lower passage should be retrieved and used. As long as metastatic potential does not change within that interval, use of cells is acceptable.

Further complicating the situation is a series of experiments which show that "trivial" culture conditions can profoundly affect metastatic potential of cells ((Welch 1997) and references therein). Examples of culture conditions that affect metastatic potential or metastasis-associated phenotypes include pH of the culture medium (Martinez-Zaguilan, *et al.* 1998), the type of medium in which the cells are grown (Prezioso, *et al.* 1993) and confluence (see (Welch 1997) and references therein).

The number of metastases in patients and in animal models is proportional to the number of tumor cells present (i.e., primary tumor size). This correlation is imperfect, but still a reasonably good rule of thumb (Glaves 1983; Hejna, *et al.* 1999; Liotta, *et al.* 1974). Recall that Tarin and colleagues demonstrated that the mere presence of tumor cells in the circulation does not always portend development of metastases. Perhaps a more critical parameter is the number or proportion of tumor cell-containing emboli in the blood (Fisher & Fisher 1967; Fisher & Fisher 1967; Lane, *et al.* 1989; Zeidman & Buss 1952). This has been shown in experimental systems. Specifically, the number and size of the emboli determined the frequency and efficiency of metastasis (Fidler 1973; Liotta, *et al.* 1976; Updyke & Nicolson 1986). These results demonstrate the need to control the inoculum, specifically, single cell suspensions should be used. To achieve predominantly single cells, the majority of clumps can be dissociated by gentle pipetting. This is maximized by the use of smaller bore pipets. However, it is important that the bore not be too small since cell killing can occur if the diameter is too small. Another condition that maximizes single cells is the use of ice-cold media or saline throughout the cell preparation steps of the procedure. The number and size of emboli increases as temperature rises. And finally, cell clumping is a time-dependent phenomenon, necessitating that inocula-containing syringes be prepared immediately before injection (Updyke & Nicolson 1986). An estimate of cell clumping can easily be determined by processing cells exactly as would be done for injection, except that they are delivered into a small dish and examined under a microscope. Single cells and clumps of various sizes can be counted directly.

Cells are loaded into the syringe when no needle is in place. The negative pressure combined with the relatively small bore of a needle causes damage and death to a relatively large number of cells. Of course, the sensitivity of cells to this particular manipulation is cell line-dependent. Until proven otherwise, it is better to be safe than sorry. As soon as the inoculum is loaded into the syringe, This makes the subsequent process of injection easier since it is easier to see the amount injected.

To obtain suspensions of mostly viable single cells, the methods used to obtain the cells from a culture are crucial. For cells growing in suspension, the technique is simple washing and dilution in an appropriate inoculation fluid (i.e., isotonic, non-allergenic). However, for adherent cultures, other methods must be employed. Scraping cells followed by gentle pipetting, filtration and/or sedimentation has been used but the yields can be inconsistent. Viability and proportion of single cells can be suboptimal using this approach (D.R. Welch, personal communication). For these reasons, enzymatic or chemical detachment are more commonly used.

The most common variations involved solutions containing the proteolytic enzyme trypsin

(0.05%-0.25%) or the chelating agent ethylenediaminetetraacetate (0.5-5 mM EDTA). Detachment times vary from less than one minute to more than one hour and this must be determined empirically. However, it is important to emphasize that exposure to any detachment agent be minimized. Prolonged treatment affects survival and metastatic potential. In the same vein, failure to remove all cells from a plate imposes a selection for weakly adherent cells which have different survival, growth and metastatic potentials than strongly adherent cells (Akiyama, *et al.* 1995; Albelda 1993; Behrens 1993; Hart, *et al.* 1991; Roos 1991; Tang & Honn 1994; Weiss 1994). And while the method of detachment may seem trivial, it is not. Metastatic potentials are profoundly affected by the conditions chosen and the magnitude and direction of the change are cell line-dependent (Welch 1997). So, until otherwise determined, conditions for cell lines received from another laboratory should not be altered. And, when a cell line is being developed and characterized, systematic evaluation of these conditions would be recommended. This notion is illustrated by a recent experience in Dan Welch's laboratory. Following transfection of human breast carcinoma cells with a candidate metastasis-suppressor gene, the cells, which are routinely subcultured using a mixture of trypsin and EDTA, became exquisitely sensitive to the presence of trypsin. In fact, even minuscule amounts of trypsin proved toxic (Md. J. Seraj and D.R. Welch, unpublished observations).

Once a properly diluted and a single cell suspension of viable cells at the "correct" level of confluence is obtained, there is yet another parameter that should be controlled. Suspensions should be maintained in polypropylene containers rather than polystyrene. Typically, tumor cells adhere better to the latter and the number of cells being injected can change as cells adhere to the walls.

Considerations regarding animals

Ultimately, the decision of which host to use is determined by the metastatic cells to be evaluated. Initially, immunologic considerations predominate the considerations. A few rules of thumb apply.

Whenever possible, syngeneic animals should be used. It is intuitively obvious that tumor development and progression are most closely recapitulated in syngeneic mice, more so in autochthonous models (Potter, *et al.* 1983; Price, *et al.* 1984). Syngeneic models are those in which tumor cell lines are derived from the same inbred strain. Autochthonous models are those in which the tumor arises within a host and the experiment is carried out in that host. The latter can be spontaneous tumors, carcinogen-induced tumors or tumors that arise in knockout, knock-in or animals. The major limitation to the use of autochthonous models relates to the large number of animals required to achieve statistically valid interpretation. This issue mostly relates to the incidence of tumors developing and the proportion of those which develop metastases. The use of genetically engineered animals may overcome these limitations (Webster & Muller 1994); however, the number of transgenic models that metastasize is still relatively limited. Table 1 lists the currently available transgenic and knockout mouse cell lines which reportedly metastasize. Readers are cautioned that this list is neither exhaustive nor does it imply a recommendation. While the use of transgene and specific gene knockouts holds great promise, a great deal more

research is required to determine the impact upon our understanding of the metastatic process. As well, more studies are required to demonstrate that the models mimic the pathobiology of human disease. Of some concern is whether more subtle aspects of the metastatic process will be discernable in these systems.

Analysis of tumor growth and metastasis of human cancers requires the use of immunodeficient animals. The most common xenograft host is the athymic mouse (*nu/nu*, T-cell deficient), also known as the "nude" mouse. Xenografts generally retain morphologic and biochemical characteristics following transplantation. Unfortunately, not all tumor types appear amenable to growth in athymic mice, making this host suboptimal. To some extent, this is alleviated with the availability of other immunodeficient strains such as the SCID (*xid*, T-cell and B-cell deficient), beige (*bg*, NK cell deficient) or mice with a combination of immune deficiencies. Intuitively, one would predict that metastatic potential would inversely correlate with relative immunodeficiency, this is not always the case (Clarke 1996; Garafalo, *et al.* 1993; Mueller, *et al.* 1991; Phillips, *et al.* 1989; Xie, *et al.* 1992). To date, there is no certain method to predict behavior in each strain.

Regardless of strain, it is crucial that all animals are tested and found to be free of infections with endoparasites (pinworm, tapeworm, ...), ectoparasites (lice, mites, ...), viruses (minute mouse virus, mouse hepatitis virus, hepatitis, pneumonia virus, ...), bacteria (*Pseudomonas*, *Staphylococcus*, ...) and *Mycoplasma*. Infections can profoundly affect experimental outcome. Therefore, sentinel animals should be tested frequently (monthly or bi-monthly) for infestation by opportunistic pathogens using sentinel animals from every animal room throughout the facility. "Routine" animal maintenance conditions (caging, light/dark cycles, diet, water chlorination, etc.) are also important and it is incumbent upon each investigator to monitor animal conditions throughout the course of the experiment.

Another consideration is natural killer (NK) cell activation. In short, metastasis has been shown to correlate inversely with NK activity (Hanna & Schneider 1983; Hanna 1982; Hanna & Fidler 1980; Hanna 1985; Urdal, *et al.* 1982). Since young mice (3 wk) have lower NK activity than older (6-8 wk) mice (Hanna, *et al.* 1982; Hanna 1982; Pollack & Fidler 1982), there is greater likelihood for observing metastases in younger, rather than older, mice. Generally, use of young mice is recommended if metastasis is a desired end point (Fidler 1986).

Site of injection

Animal models for metastasis typically involve two approaches. The first involves inoculation of tumor cells into tissue sites (i.e., subcutaneously (s.c.), intradermally (i.d.), intramuscularly (i.m.), or into specific organs or tissues (e.g., mammary fat pad (m.f.p.)) which results in the formation of a local tumor from which *spontaneous* metastases eventually form. The second approach bypasses local tumor growth and intravasation by introducing tumor cells directly into the vasculature (usually intravenously (i.v.), but also intra-arterially (i.a.) or intra-cardially (i.c.)). This results in formation of *experimental* metastases. Both methods have contributed to our understanding of the multigenic, multistep metastatic phenotype; however, the experimental metastasis assay has been maligned by some. While there are valid reasons for

questioning the direct vascular injection of tumor cells (reviewed in (Welch, *et al.* 1983; Welch 1997), there are equally cogent assertions that this model is appropriate. It is crucial, however, to establish whether results from both assays are equivalent. An example where distribution of metastases is significantly different depending upon route of injection involves use of the MDA-MB-231 human breast carcinoma cell line. Intravenous injection produces lung metastases with occasional extrapulmonary metastases. However, inoculation directly into the left ventricle results in formation of osteolytic metastases in the long bones, a condition rarely seen in mice (Guise & Mundy 1998; Rabbani, *et al.* 1999; Yang, *et al.* 1999; Yin, *et al.* 1999; Yoneda, *et al.* 1994), but common in human breast carcinomas.

Some argue that bolus inoculation of thousands of tumor cells directly into the vasculature does not reflect the situation in humans; however, quantification of tumor cells in patient blood indicates that numbers between 10^4 to 10^7 are not unreasonable (Tarin, *et al.* 1984; Willis 1973). Indeed, mere presence of large numbers of tumor cells in the blood does not necessarily mean that macroscopic metastases will develop (Luzzi, *et al.* 1998; Tarin, *et al.* 1984; Weiss 1990). Perhaps the more relevant concern is the condition of the tumor cells at the time of injection and the site at which cells enter the vasculature.

Although injection of tumor cells into the vasculature results in wide distribution of tumor cells throughout the body (Chan, *et al.* 1988; Juacaba, *et al.* 1989; Potter, *et al.* 1983), the most cells interact with and are arrested in the first capillary bed encountered. It follows, then, that the site of injection can be used to enhance development of metastases at a particular organ. This strategy has been taken advantage of during the selection of subpopulations with increased propensity to colonize a particular organ (Chambers, *et al.* 1982; Chambers & Wilson 1988; Fidler 1973; Giavazzi, *et al.* 1986; Kawaguchi, *et al.* 1983; Miner, *et al.* 1982; Nicolson, *et al.* 1989; Sargent, *et al.* 1988). Intravenous inoculation into the lateral tail vein is the most common route of injection for the experimental metastasis assay. As expected, the typical site colonized is at the first capillary bed encountered – lung.

A great deal of attention has been recently afforded the orthotopic injection of cancer cells. Even a cursory review of the literature shows that most investigators have injected tumor cells subcutaneously. The reasons are simple – injections are convenient, tumor monitoring and measuring are convenient and skill level required is minimal. For a substantial number of tumorigenicity studies, more complicated protocols are not necessary. However, most tumors fail to metastasize from this site (Liotta 1986), despite maintaining morphological and biochemical characteristics. It is logical to contend that injection into an orthotopic site is more relevant and some investigators contend that orthotopic implantation is essential (Fidler 1990; Fidler 1991; Hoffman 1994; Kerbel, *et al.* 1991; Kubota 1994; Meyvisch 1983). Orthotopic implantation (colorectal carcinoma — caecum (Bresalier, *et al.* 1987; Dong, *et al.* 1994; Fidler 1991; Fu, *et al.* 1991; Morikawa, *et al.* 1988; Singh, *et al.* 1997), renal carcinoma — kidney or subrenal capsule (Clayman, *et al.* 1985; Naito, *et al.* 1986; Naito, *et al.* 1982; Singh, *et al.* 1994), cutaneous melanoma — intradermal (Juhasz, *et al.* 1993; Miele, *et al.* 1996; Welch, *et al.* 1991), ocular melanomas — intra-ocular or choroidal (Albert, *et al.* 19880; Niederkorn, *et al.* 1981), bladder carcinomas — bladder wall (Ibrahiem, *et al.* 1983; Kawamata, *et al.* 1995a; Kawamata, *et al.* 1995b; Kerbel, *et al.* 1991; Theodorescu, *et al.* 1991; Theodorescu, *et al.* 1990), breast

carcinoma — mammary fat pad (Bao, *et al.* 1994; Kaufmann, *et al.* 1996; Levy, *et al.* 1982; Miller & McInerney 1988; Phillips, *et al.* 1996; Price 1996; Price & Zhang 1990; Price, *et al.* 1990), prostatic carcinoma — prostate (Dong, *et al.* 1999; Knox, *et al.* 1993; Rembrink, *et al.* 1997; Stephenson, *et al.* 1992; Yang, *et al.* 1999), pancreatic carcinoma — pancreas (An, *et al.* 1996; Marincola, *et al.* 1989; Reyes, *et al.* 1996; Tan & Chu 1985) , osteosarcomas — bone (Berlin, *et al.* 1993; Crnalic, *et al.* 1997; Simon, *et al.* 1998), gastric adenocarcinoma — stomach wall (Fujihara, *et al.* 1998; Togo, *et al.* 1995), lung tumors — intrabronchial or intrapleural (Howard, *et al.* 1991; McLemore, *et al.* 1987; Nagamachi, *et al.* 1998)) often results in greater metastatic efficiency and more relevant colonization patterns (i.e., similar to human cancer) than ectopic implantations into age- and sex-matched mice. Hoffman and colleagues further contend that surgical implantation of tumor fragments increases metastatic potential compared to inoculation of single cells into an orthotopic site (Hoffman 1994). The mechanisms for the orthotopic effect remain largely unknown, but insights are forthcoming (Hoffman 1994; Kerbel, *et al.* 1991; Singh, *et al.* 1997).

Another common occurrence is injection of tumor cells into multiple sites of the same animal. This practice may complicate interpretation since tumor cells communicate with each other and profoundly influence the biological behavior of distant cells. Several examples are described in the clinical and experimental literature (Brunschwig, *et al.* 1965; Clark, *et al.* 1989; Fidler & Lieber 1972; Fisher, *et al.* 1989; Howard 1963; Koike, *et al.* 1963; O'Reilly, *et al.* 1994; Southam & Brunschwig 1961; Sugerbaker, *et al.* 1977; Warren, *et al.* 1977; Woodruff 1990; Woodruff 1980)or inhibit (Gorelik, *et al.* 1982; Isoai, *et al.* 1990; Torosian & Bartlett 1993). So, unless cell-cell communication is being tested, *in vivo* studies should not employ inocula into multiple sites of the same animal.

Materials Needed

All of the materials needed for these studies are available through a variety of scientific product distributors. Unless specifically warranted, specific brand names are not recommended since they vary considerably according to availability. Rather, key manufacturing criteria (e.g., construct material or components) are provided.

It is advised that all the cell inoculum be prepared with 25-50% more volume than the amount calculated to be necessary. During the course of injections, volume is lost. Rather than cutting it close, it is advisable to prepare extra. Also, during the injection process, trituration of the cell suspension (with the needle absent) is encouraged to minimize cell clumping or sedimentation.

Spontaneous metastasis assays

There are so many variations of the spontaneous metastasis assay that it is impossible to describe all of them here. Therefore, two approaches are provided as examples from which the reader is to extrapolate to his/her situation. Once cells are prepared according to the criteria outlined above, the next decision is site of injection. Unless a *bona fide* scientifically-based

reason is presented, orthotopic injection is recommended. The two examples provided will be injection of melanoma cells intradermally and breast carcinoma cells into the mammary fat pad. In both cases, the experiments outlined involve mice, but other animals can be used as warranted by the model.

There are two common variations of the spontaneous metastasis assay: (1) animals in which the primary tumor remains throughout the experiment; and (2) animals in which the primary tumor is removed (to allow time for metastases to grow to detectable size). With regard to the former, it is becoming increasingly common that animal facilities will not allow tumors to achieve sizes ≥ 1 cm. Under these conditions, the likelihood of developing metastases, especially in xenograft models, is low. The honorable intention of avoiding suffering has an undesirable byproduct for the metastasis researcher – studies of late-stage tumor biology becomes increasingly difficult, perhaps impossible. Therefore, it is important that two things take place. First, institutional animal care and use committees should be educated regarding this issue. Emotional arguments must be countered with rational, persuasive, yet still compassionate, polemic. Second, appropriate safeguards should be introduced so that euthanasia or treatment is initiated at the first sign of distress.

Some of these issues are alleviated when the primary tumors are surgically removed. Timing of this operation is critical. Since tumor size is proportional to the likelihood of developing metastases (Price, *et al.* 1990; Safarians, *et al.* 1996), one must balance probability that metastases will have developed with the complexity of the surgery. Generally, a mean tumor diameter (square root of the product of orthogonal measurements) or geometric tumor diameter (cubed root of the product of three orthogonal measurements, distinct from mean tumor diameter because depth is measured) of 1.0-1.5 cm achieves the balance. If metastases are going to develop, they will likely have done so once a tumor has reached this size. And, even in small mice, a tumor can be removed without difficulty or post-surgical complications.

The choice to focus on intradermal and mammary fat pad injection also allows a discussion of other anatomic considerations. While obvious for skin, it is less well known that the mammary fat pads are extensive and span nearly the entire length of the ventral surface of mature rats and mice. There are even vestiges of mammary tissue on the flanks and backs of rats and mice. Orthotopic introduction of breast tumor cell lines into these animal could, therefore, be done over a wide area. Nonetheless, assurance that one is actually injecting into the mammary fat pad is easiest near the teats. Which teat? Kyriazis and Kyriazis (Kyriazis & Kyriazis 1980) indicate that there is a so-called "cranial-caudal" gradient that influences tumor behavior. MDA-MB-435 human breast carcinomas metastasize more often from the thoracic mammary fat pad than from the inguinal mammary fat pad (Meschter, *et al.* 1992). The pattern of metastases also changed – tumors in the inguinal region produced extensive intra-abdominal lymph node metastases; whereas, thoracic tumors developed more blood-borne metastases. Similar findings with melanoma cells have been described (Bani, *et al.* 1996; Welch 1997). Clearly, the frequency and location of metastasis is altered based upon implantation distance from the head (Price 1996). Placement also will impact the ability to remove the locally growing tumor if needed.

Inoculation volumes vary according to the site of injection. Maximum inoculum volume should not exceed 100 µl for mammary fat pad injections, 50-100 µl for intrasplenic injections,

25-50 µl for intraadrenal injections or 50-100 µl for intradermal injections. Subcutaneous injections can utilize volumes as high as 0.5 ml. Keeping the volume below these levels minimizes leakage into surrounding tissues and stromal and epithelial damage. On the other hand, if the volume is too small, accuracy of injection becomes difficult to control. For volumes less than 100 µl, accuracy of delivery volume is best accomplished with a sterile Hamilton syringe or tuberculin syringes. The viscosity of solution being inoculated is also important. It is therefore recommended that cell concentrations no greater than 1×10^8 cells/ml be used. In addition, it is important that the inoculum not leak from the needle tract. While this is not always easy to insure, some precautions should instituted to minimize the possibility.

Injections into the subcutaneous or intradermal sites do not require that the animals are anesthetized; however, intradermal injections are significantly easier when the animals are unconscious or sedated. Metastatic potential does not seem to be affected by anesthetics at this step. Methoxyflurane (Metofane®, Pitman-Moore) inhalation anesthetic is relatively inexpensive and effective for this purpose. A homemade anesthesia jar will suffice. Place a small volume of Metofane® under a wire screen suspended above the floor of a container with a lid. Mice and rats will be unconscious within a 3-5 minutes and the effects will last less than 5 minutes. It is crucial that the animals never come into contact with the fluid. Transdermal absorption can be lethal. Also, anyone using this inhalant should also be cognizant of the content within the Material Safety Data Sheets (MSDS) and the chances of pathologies if overexposed. Hint: construct the anesthesia jar with a wire mesh that allows feces to drop as this maintains clean conditions. This provides for easier and more complete cleaning of the jar.

For injection into other sites (mammary fat pad, intrasplenic, other orthotopic sites), a mixture of Ketaset-Rompun (Ketamine-HCl, xylazine) injected intramuscularly provides excellent results. This anesthetic is also useful for simple surgical procedures like tumor removal. A stock solution of 10 ml Ketamine (100 mg/ml) containing 1.6 ml xylazine (20 mg/ml) works well for rats. A female Fisher 344 rat weighing 150-180 gm inoculated i.m. with 0.1-0.15 ml will remain unconscious for 1-4 hr. For mice, the stock solution should be diluted 1:10 in saline. For most mice, we have found that 0.1 ml/10 g body weight is sufficient to anesthetize for 30 min to 1 hr. However, nude mice require a higher dose 0.15 ml/10 g body weight). The reasons for this difference in dosage are not completely understood.

Figures 2 and 3 show intradermal and mammary fat pad injections, respectively. For photographic purposes, the animals were also sedated for intradermal injections. Both types of injection used 27 gauge needles attached to tuberculin 1 cc syringes. The single cell suspension was prepared in ice-cold Hank's Balanced Salt Solution (however, any isotonic liquid will work as long as it does not contain serum). Note that the bevel faces the syringe markings, making it easy to see injection volume. Also note that for all of the injections the bevel is oriented so that the bore is visible from the top.

For the mammary fat pad injection, a small incision is made toward the midline of the teat using scissors (a scalpel works also) (Figures 2A and 2B). The mammary fat pad is exposed in the incision by inverting the tissue using a finger and sliding the needle into the fat pad immediately under the teat (Figure 2C). A "blister" forms at the site of injection that is translucent and relatively fragile. For this type of injection, slower injection rates are

recommended. When the inoculum has been injected, the needle is withdrawn and the incision is closed with sterile wound clips (Figure 2D). Removal of the wound clips is usually not necessary since they usually fall out with 1-2 weeks. If, however, the clips remain, they pose no adverse threat to the animal or the tumor.

Intradermal injections are similar to mammary fat pad injections in many respects (Figure 3). Photographically, the intradermal injection is indistinguishable from a subcutaneous injection. The needle is inserted into a skin fold and then reintroduced into the skin from the internal surface. For a subcutaneous injection, the needle is simply placed into the skin fold. A hallmark of intradermal injection is greater resistance during the process. Subcutaneous injections seldom require much pressure to be placed on the plunger.

If the local tumor (sometimes called the "primary" tumor) is to be removed, the animals are anesthetized using the same Ketamine-Rompun solution described above. Adequate sedation is easily determined by pinching a foot between ones fingers. If the animal responds, it is not adequately sedated. Tumors are removed with a wide margin (highly variable) with scissors or a scalpel. At this stage premeditation is key in order to produce a cosmetically and medically acceptable result. Deliberate maneuvers utilizing the fewest possible cut angles is best. This leaves smooth edges which are less prone to infection and which heal faster. The wounds can be sutured (consult a veterinarian for advise regarding the best composition) or stapled with sterile wound clips. Depending upon the facility and the site, topical antibiotics can be applied to further decrease the chances for infection. Standard aseptic techniques are typically all that is necessary for infection not to be a problem. Dissolving sutures is recommended in order to minimize follow-up anesthesia. This is particularly desirable if visceral tumors are removed. Wound clips are not recommended for internal wound closure. It is not necessary to remove them as they tend to fall out shortly after healing is complete anyway.

Experimental metastasis assays

The mechanics for experimental metastasis assays are similar to those for the spontaneous metastasis assay. As above, volume and viscosity of the inoculum are important parameters. Volumes should never exceed 0.2 ml because plasma blood volume exceeds the normal range (± 10%) such that the distribution pattern of cells is altered. Tumor density is even more critical for intra-vascular injections since introduction of emboli can cause death because of vascular obstruction. Therefore, the same concentration maximums are also applicable for this type of assay.

The key difference for intravenous injection is the use of a restrainer in which allows the tail to extend outside of the enclosure for i.v. injections. Several restrainer designs are available. The most common are plexiglass. The preferred design is a hinged stainless-steel tube suspended on a weighted pedestal (Please note that the restrainer shown (Figure 1A) is custom made for D.R. Welch.). This unit is autoclavable; however, rinsing with a dilute bleach solution suffices for sterilization. A mouse is placed into the tube and enclosed while holding the tail (Figure 1E). The dark environment has a calming effect (A similar effect can be obtained in the plexiglass restrainers by wrapping with electrical tape and attaching a piece of lead to the bottom of the

restrainer to increase the weight in order to minimize movement during the injection process.). A lateral tail vein is identified and the needle is gently inserted to just below the skin. After "tracking" for some distance to minimize leakage and backwash when the needle is withdrawn, the cells are injected using a 27 gauge needle fitted onto a 1 cc tuberculin syringe (some prefer a 26 gg needle) (Figure 1E). During the process, a slow, steady rate of injection is the objective. For i.v. injections, successful inoculation is evident by the lack of resistance during the process. If any resistance is felt, the process should be restarted. It is best to begin injections at the most distal part of the tail. If the injection is missed, one can proceed cranially. If one starts at the base of the tail, the effort cannot be redeemed without subsequent inocula leaking from the hole(s) generated by prior injections. Although i.v. inoculations can be done without the aid of procedures to dilate tail veins, the process is facilitated when tail veins are enlarged. This can be accomplished by dipping the tails into hot water, swabbing with irritants (such as xylenes) or brief warming under a heat lamp (preferred). A high-walled container is useful in order to decrease the chances for mice escaping (Figure 1B). This is a particular concern for "hyperactive" mice such as C57BL/6. Use of a heat (infrared) lamp requires close monitoring to assure that the animals do not get overheated and that personnel are careful not to touch the bulb since second and third degree burns can result.

Enumeration of metastases

After injecting the tumor cells. The hardest part of the experiment follows – waiting. The interval can be a few days for particularly aggressive cells to several months for others. When a new model is being developed, it is important to allow adequate time for metastases to develop. Therefore, periodic euthanasia of a subset is advised. If, at any time, animal health appears to diminish, the experiment should be terminated for humane reasons.

For some studies, the mere presence or absence of metastases is sufficient; however, quantification of metastases is often desired. To assess metastasis, animals are killed, organs are examined then removed and rinsed in cold water to remove excess blood.

Euthanasia methodology affects the ease by which metastases can be detected and quantified. Three methods have been used by my laboratory — carbon dioxide asphyxiation, cervical dislocation and overdose using anesthetics. All methods for euthanizing animals must be approved beforehand by Institutional Animal Care and Use Committees (IACUC). Guidelines for euthanasia are evolving; so, regular consultation with veterinarians concerning procedures is advised.

Carbon dioxide is not recommended because of a substantial number of petechia in the lungs. They complicate counting of tumors, particularly for less inexperienced lab personnel. Cervical dislocation works well but sometimes can result in the presence of clots in the lungs. Nonetheless, cervical dislocation is still used as the primary method for mouse euthanasia (See below). For rats and as an alternative for mice, Metofane inhalation works extremely well. The animals fall asleep and then die. Lungs are clear and quantification of metastasis is unimpeded . However, this is more expensive.

Identification of macroscopic metastases is easier if coloration is different from parenchyma.

This was a major advantage when studying melanoma; however, this characteristic was not available for other tumor types. It is possible to identify metastases in most tissues. They appear as clear or white raised gelatinous surface structures. Examination is facilitated by the use of a dissecting or stereomicroscope. However, if there are imperfections in the tissue or if the tumor is small, errors can occur. Therefore, different approaches have been employed to enhance visual contrast between tumor and parenchyma.

For lung metastases, the trachea can be injected with 1.5-2.5 ml of a 15% solution of India ink in neutral buffered formalin (37% formaldehyde (100 ml), tap water (900 ml), sodium phosphate monohydrate (4 g), disodium phosphate (6.5 g), pH 7.0). Following sealing with surgical suture to prevent leakage; the lungs are then suspended in a beaker of tap water. Tumor colonies are then bleached with Fekete's solution (37% formaldehyde (10 ml), glacial acetate (5 ml), 70% ethanol (100 ml)). Metastases appear as white colonies against a black background. While this approach works well, it can be impractical when lots of animals are being necropsied simultaneously. Also, it can be done more easily with assistance. Therefore, the preferred method is fixation of organs and tissues in Bouin's solution (saturated picric acid (300 ml), neutral buffered formalin (100 ml), glacial acetate(20 ml)). After fixation, the tumors appear as white or pale yellow spots against a darker yellow background. The use of Bouin's is not without problems, however. Tissues become brittle making subsequent confirmatory histology difficult. To partially alleviate this problem, we use a mixture of 1 part Bouin's fixative in 5 parts neutral-buffered formalin.

One important consideration when removing tissues is to avoid touching the surface with forceps. Striations caused by the teeth complicate visualization of small metastases. Most lung (Polissar & Shimkin 1954; Welch, *et al.* 1983; Wexler 1966; Wood, *et al.* 1954) and liver (Lefreniere & Rosenberg 1986) metastases develop near the surface; therefore, anything which compromises visualization will affect quantification. Similar precautions should be taken during the preparation of other tissues.

Random samples of tissues should be submitted for histologic confirmation of presence/absence of metastases. Ideally, one could quantify metastasis by serially sectioning tissues, measuring the surface area of the lesions, calculating total tumor volume and comparing that to the organ/tissue volume (Boeryd, *et al.* 1966). Unfortunately, this is impractical. The use of histologic preparations allows determination of total tumor burden. Most investigators merely count the number of metastases. Small lesions are not equivalent to large lesions, however. Assessment of metastatic tumor burden should not only include assessment of number, but also size/volume of lesions. In the lung, the majority of metastases are spherical (Welch, *et al.* 1983) making this calculation straightforward. Side-by-side comparison of the number and volume of metastases can give important information regarding mechanism responsible for developing metastasis by different cell lines.

Although well established, counting and measuring metastasis can be tedious, especially for large-scale experiments such as drug screening for antimetastatic compounds. Organ weight can offer an option. However, care must be taken to properly control for experimental read-out.

First, organ weight is not a valid measure alone since this is proportionate to animal weight/mass. Therefore, a ratio of organ weight to animal weight should be calculated. Second,

measurements must accompany verification that ancillary variables have not caused changes. For example, some treatments can cause edema which would increase organ weight thereby mimicking tumor burden. Organ weight and metastatic tumor burden correlates well in some tumor models (e.g., Lewis lung carcinoma (Gorelik, *et al.* 1980), but not in others (Welch 1997). Therefore, use of organ weights as a measure of metastasis must be confirmed on a case-by-case basis.

Welch has considered development of image analysis software for quantification of metastasis (D.R. Welch, personal communication). While this would greatly aid the metastasis researcher, three limitations remain – variable "staining" of the metastases; difficulty distinguishing organ surface imperfections from *bona fide* small metastases; and three-dimensional nature of organ surfaces. Staining conditions could be worked out with systematic evaluation. The imperfection issue is more difficult because such imperfections are not predictable or consistent, making programming very difficult. Finally, image analysis is dependent upon two-dimensional photographic or imaging systems. Unless multiple images from different angles are prepared and assimilated using computer algorithms, this is not likely to be cost-effective in the short term.

Another method for estimating the number of metastatic cells successfully colonizing a tissue is accomplished using tumor cells tagged with a drug resistance, genetic or color marker. Visualization of metastases can be enhanced if the cells are tagged with the lacZ gene (Brunner, *et al.* 1992; Brunner, *et al.* 1993; Fujimaki, *et al.* 1993; Kurebayashi, *et al.* 1993; Lin, *et al.* 1990; McLeskey, *et al.* 1996) or fluorescein (Potter, *et al.* 1983). The tumor cells appear blue or yellow-green, respectively. However, stability of the transfectants *in vivo* can vary considerably and a substantial proportion of macroscopic metastases will no longer be colored or the metastases will contain mixtures of colored and colorless cells. Therefore, if coloration were used as a criterion, the number and volume of metastasis would be underestimated (Fujimaki, *et al.* 1993). If tumor cells are tagged with a drug resistance or genetic marker, cells colonizing different organs can be recovered from dissociated tissues (Miller, *et al.* 1990) and the proportion compared to the inoculum. Similarly, cells labeled with ^{125}IUdR or BrdU can be detected in dissociated tissues using a gamma counter or ELISA, respectively (Fujimaki, *et al.* 1993). To use these approaches, prior verification that the label does not affect biological behavior of cells must be obtained.

Cells transfected with enhanced green fluorescent protein (GFP (Cubitt, *et al.* 1995)) are exposed to blue light and the tumor cells fluoresce in the green range. This approach was first utilized by others to visualize metastases *in vivo* (Chishima, *et al.* 1997; Chishima, *et al.* 1997; Farina, *et al.* 1998; Yang, *et al.* 1999; Yang, *et al.* 1998) and offers a significant increase in sensitivity since metastases are more visible. However, a caveat to these techniques deserves mention. Presence of single cells in an organ should not be equated with metastasis formation. Clinically relevant metastases are those which have grown to sufficient size to disrupt cellular or tissue function. This is usually not the case with single cells. As such, single cells do not qualify as having completed all steps in the metastatic cascade. That being said, single cells can remain as occult disease for several years until stimulated to divide (i.e., complete the metastatic cascade). Presence of single cells should not be ignored, but should be categorized differently

than macroscopic metastases.

Statistical considerations

New investigators are often shocked by the relatively high variability observed when doing *in vivo* studies. While it should not be surprising, given the increased number of variables incorporated into the experimental design, the "shock" that accompanies an inadequately designed experiment is real. It is critical, therefore, that each experiment include sufficient "power" to provide a statistically valid result. For most studies, groups of a minimum of 8-10 animals are required, but sometimes more are needed. The total number of test animals needed can be determined using appropriate power calculations (Heitjan, *et al.* 1993).

As with most statistical calculations, increasing the number of measurements increases the likelihood that one will obtain normally distributed data. If this is the case, then parametric assays can be used. However, for many studies, non-parametric statistics are necessary. The most common reasons for needing non-parametric tests are non-normal distribution of low metastasizing cell lines (i.e., the numbers of metastases are mostly zero) or counts greater than a countable number (i.e., >250 metastases per lung). In both cases, the data are not normally distributed. In this situation, the statistic used is the Mann-Whitney U-test which evaluates differences between groups using ranks. Readers are encouraged to consult with a statistics textbook and/or a statistician prior to beginning a study and when interpreting the results.

Concluding remarks

Metastasis assays are, by their very nature, complicated. This is largely because the process of metastasis is itself complex and variable. The key elements to successfully studying metastasis are careful consideration of the question(s) being asked, quality characterization of the cell lines being used for the study, and utilization of appropriate model(s). The technical components of the studies require due care, but attention to the details will enhance the likelihood of success.

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Figure Legends

Figure 1: Depiction of the set-up and intravenous injection of tumor cells for the experimental metastasis assay. Panel A shows a restrainer sitting atop a sterile surgical pad. Sufficient needles and syringes are opened and immediately available to complete all of the

injections. To the right of the restrainer is a cage which sits underneath an infrared light used to cause dilation of the tail veins (Panel B). The syringe is loaded with cell suspension when there is no needle attached to the syringe (Panel D). Following syringe loading, the needle is placed with the bevel opening aligned with the syringe markings (Panel C). The lateral tail vein is located, visualized and the needle is inserted under the skin and "tracked" up the vein (not the dark vein). Tail coloration in this photograph is the result of room lighting conditions and proximity to the heat lamp. Tail reddening should never occur.

Figure 2: Key steps involved in orthotopic mammary fat pad injections. An incision is made medially to the teat under which the fat pad will be exposed (Panels A and B). The fat pad is externalized by inverting the skin using a finger (Panel C). The needle is inserted into the fatty tissue below the teat and inoculum injected. Panel D shows the mouse following closure of the incision with wound clips. As noted in Figure 1, coloration is distorted due to room lighting, flash and sterile hood lighting conditions.

Figure 3: Intradermal injection of cells into the athymic mouse. The needle is inserted subcutaneously and then tracked to injected into the skin from the internal side. A bleb indicates that the inoculate is being injected.

Figure 4: Visualization of lung metastases following staining with Bouin's fixative (lighter colored nodules on the surface, Panel A, two lungs to the right of the normal lung) or following injection of India ink solution into the trachea followed by destaining with Fekete's solution (Panel B depicting white nodules against the background.

Table 1: Transgenic and knockout mice which develop metastases		
Transgene or knockout	**Site of primary tumor**	**Reference(s)**
Review of transgenic metastasis models	mammary	(Dankort & Muller 1996)
$Nf2^{+/-}$	multiple	(McClatchey, *et al.* 1998)
$p27KIP1^{-/-}$	prostate	(Cordon-Cardo, *et al.* 1998)
$p53^{-/-}$ + TGF-β1	skin	(Akhurst & Balmain 1999)
tg: SV40T	prostate	(Foster, *et al.* 1997; Gingrich, *et al.* 1997; Gingrich, *et al.* 1996)
tg: × SCID, beige (immune-deficient mice)	pancreas	(Gallo-Hendrikx, *et al.* 1994)
tg: cryptdin-2-SV40T	prostate	(Garabedian, *et al.* 1998)
tg: Metallothionein - Ret	melanoma	(Asai, *et al.* 1999)
tg: MMTV-LTR-mts1 × GRS/A	mammary	(Ambartsumian, *et al.* 1996)
tg: polyomavirus middle T	mammary	(Ritland, *et al.* 1997)
tg: MMTV-Fgf8b	mammary, salivary gland	(Daphna-Iken, *et al.* 1998)
tg: Tyr-SV40E + UV irradiation	melanoma	(Kelsall & Mintz 1998)
tg: Tyr-SV40T	retinal pigment epithelium	(Penna, *et al.* 1998)
tg: fetal globin-SV40T	prostate	(Perez-Stable, *et al.* 1997)
tg: Rip1Tag2 × E-cadherin dominant negative	pancreatic	(Perl, *et al.* 1998)
tg: RET/PTC3	papillary thyroid	(Powell, Jr., *et al.* 1998)
tg: MMTV-neu	mammary	(Ritland, *et al.* 1997)
tg: MMTV-polyoma middle T	mammary	(Ritland, *et al.* 1997)
tg: MMTV-polyoma middle T	mammary	(Lifsted, *et al.* 1998)
tg: keratin-$p53^{172H}$	keratinocyte	(Wang, *et al.* 1998)
tg: Probasin- SV40T	prostate	(Kasper, *et al.* 1998)
tg: C3(1)-SV40T × $p53^{-/-}$	prostate	(Maroulakou, *et al.* 1997)
tg: HGF/SF	melanoma	(Otsuka, *et al.* 1998)

Note: Several papers utilize tumor cell implants into knockout or transgenic mice with associated changes in metastatic potential (Araki, *et al.* 1997; Biancone, *et al.* 1996; Bourguignon, *et al.* 1998; Davies, *et al.* 1996; De Vries, *et al.* 1995; Driessens, *et al.* 1995; Eitzman, *et al.* 1996; Goldfarb, *et al.* 1998; Hall & Thompson 1997; Kruger, *et al.* 1998; Lloyd, *et al.* 1998; Marvin, *et al.* 1998). The citations listed above are those in which metastases are observed in the genetically engineered mice without inoculation of tumor cells.

Table 2: Commonly used metastasis models

Cell Line	Route(s) of Injection	Organ tropism	Species	Reference(s)
B16 melanoma				
B16-F10	i.v., s.c., i.m.	Lung, brain, ovaries, intestine	mouse, C57BL/6	(Fidler 1973)
B16-F1	i.v., s.c., i.m.	Lung	mouse, C57BL/6	(Fidler 1973)
B16-O10	i.v.	Ovary, lung, other	mouse, C57BL/6	(Brunson & Nicolson 1979)
B16-B15b	i.v. (carotid)	Meninges, lung, other	mouse, C57BL/6	(Kawaguchi, *et al.* 1983; Miner, *et al.* 1982)
B16-BL6	i.m. (With leg amputation)	Lung	mouse, C57BL/6	(Poste, *et al.* 1980)
13762A mammary	i.p.	Lymph node, ascites	rat, F344	(Osbakken, *et al.* 1986)
13762NF mammary	m.f.p., i.v.	Lung, lymph node	rat, F344	(Neri, *et al.* 1982; Welch, *et al.* 1983)
C8161 melanoma	i.v., i.d.	Lung, brain, liver, ovaries	human (nu/nu, SCID)	(Welch, *et al.* 1991)
Co-3 colon	colon	liver	human (nu/nu)	(An, *et al.* 1997)
Dunning R3327 prostate	s,c,	lung, lymph node	rat, Copehnagen	(Isaacs, *et al.* 1978)
Esb/Eb lymphoma	s.c.	Liver, lung, spleen	mouse, DBA/2	(Altevogt, *et al.* 1985)
F9 teratocarcinoma	i.v.	Liver, lung	mouse, C57BL/6	(Cotte, *et al.* 1982)
HRCC	kidney	lung	human (nu/nu)	(Naito, *et al.* 1986)
K1735 melanoma	i.v.	Lung	mouse,	(Volk, *et al.* 1984)
Lewis lung carcinoma (3LL)	s.c., i.v.	Lung	mouse, C57BL/6	(Giraldi, *et al.* 1977; Gorelik, *et al.* 1980; Gorelik, *et al.* 1982; Hilgard, *et al.* 1976; Young, *et al.* 1990)
LOX melanoma	i.v., s.c., i.d.	Lung	human (nu/nu)	(Shoemaker, *et al.* 1991)
M24met melanoma	i.v., s.c.	Lung	human (nu/nu, SCID)	(Mueller, *et al.* 1991)
M4Be melanoma	s.c.	Lung	rat (nu/nu)	(Bailly & Dore 1991)
MDA-MB-231	i.c.	Bone	human (nu/nu)	(Sasaki, *et al.* 1995)
MDA-MB-231	i.v.	Lung	human (nu/nu)	(Price, *et al.* 1990)

MDA-MB-435 breast	m.f.p.	Lung, lymph node	human (nu/nu)	(Price, *et al.* 1990)
MDAY-D2 lymphoma	s.c., i.v.	Liver, lungs, spleen, kidney	mouse, DBA/2	(Kerbel, *et al.* 1978)
MelJuSo melanoma	i.d., i.v.	Lung	human (nu/nu)	(Miele, *et al.* 1996)
MeWo melanoma	i.v., s.c.	Lung	human (nu/nu)	(Ishikawa & Kerbel 1989; Ishikawa, *et al.* 1988)
MMTV mammary tumors	s.c., i.v., m.f.p.	Lung, liver, lymph node	mouse, BALB/c	(Heppner, *et al.* 1978; Miller 1981)
P574 mammary	spontaneous	Lung, adrenal, ovary, kidney	mouse, C3H	(Price, *et al.* 1984; Tarin & Price 1981)
PAN-12 pancreas	pancreas	liver, kidney, lymph node	human (nu/nu)	(An, *et al.* 1996)
ras-transfected NIH-3T3 fibrosarcoma	i.v.	Lung	mouse (nu/nu)	(Chambers & Tuck 1988)
RAW117 lymphosarcoma	i.v., s.c.	Liver, spleen	mouse, BALB/c	(Brunson & Nicolson 1978)
SP1 mammary	s.c.	Lung	mouse, CBA	(Frost, *et al.* 1987)
UV2237 fibrosarcoma	i.v.	Lung, skin, mesentary	mouse, C3H/HeNCr	(Kripke, *et al.* 1978)

The cell lines listed in this table are not complete, particularly with regard to variants selected from the parental. Since the B16 melanoma is the most commonly used metastatic cell line used, the most popular variants are listed with associated references.

Abbreviations used: i.v., intravenous; s.c., subcutaneous; i.c., intracardiac; m.f.p., mammary fat pad; i.d., intradermal; i.p., intraperitoneal

Note: This list of tumors is not exhaustive and is provided only as a resource to identify some commonly used metastatic tumor cell lines.

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Correlation Between Reduction of Metastasis in the MDA-MB-435 Model System and Increased Expression of the Kai-1 Protein

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Using microcell-mediated transfer of a normal chromosome 11 into the highly metastatic MDA-MB-435 human breast carcinoma cell line, we previously showed that human chromosome 11 contains a metastasis-suppressor gene for breast cancer. A known metastasis-suppressor gene, *kai-1*, and a related family member, *tapa-1*, have been mapped to chromosome 11p11.2 and 11p15.5, respectively. To determine if these genes are responsible for the metastasis suppression seen in our microcell hybrids, we examined their expression by western blot analysis. Although tapa-1 expression did not significantly correlate with metastasis suppression, kai-1 production was dramatically increased in the metastasis-suppressed chromosome 11 microcell hybrids and unchanged in the metastatic chromosome 6 controls. Transfection of full-length *kai-1* cDNA into MDA-MB-435 cells resulted in clones that did not have a significantly decreased in vivo incidence of lung metastases. However, western blot analysis showed that the primary tumors and the metastatic lesions of the transfectants had decreased levels of kai-1 protein compared with the inoculated cells. Furthermore, several of the transfectant clones expressed heavily modified kai-1 protein compared with that of the microcell hybrids. Our data indicate that protein modification may affect the normal function of kai-1 in vivo and that a threshold level of kai-1 protein expression may be necessary for suppression of the metastatic phenotype. *Mol. Carcinog. 21:111–120, 1998.*

Key words: metastasis; suppressor gene; breast cancer; human chromosome 11; microcell hybrid

INTRODUCTION

Loss of heterozygosity (LOH) studies have implicated several genes on chromosome 11 in the formation of many tumor types, including breast [1–3], cervical [4], lung [5,6], nasopharyngeal [7], and ovarian carcinoma [8], as well as neuroblastoma [9] and malignant melanoma [10]. Furthermore, a tumor-suppressor gene for Wilms' tumor and a metastasis-suppressor gene for prostate cancer map to chromosome 11p13 and 11p12, respectively [11–13], and the 11p15.5 region is under investigation for a second sporadic Wilms' tumor gene [14,15]. Additionally, the α2-integrin gene and the *ATM* (ataxia telangiectasia gene), which both map to chromosome 11, have been implicated in malignancy [16,17]. Therefore, studies that help to define the importance of genes on chromosome 11 have been among the most important for determining molecular events leading to neoplasia. Indeed, to fully comprehend the mechanisms involved in tumor progression, it may be critical to identify changes in gene expression on chromosome 11 for all cancer types.

Different stages of cancer may also be controlled by different genes on chromosome 11. For example, cytogenetic analyses of metastatic breast tumors have shown that chromosome 11 rearrangements often appear late in breast cancer [18], suggesting the presence of a metastasis-suppressor gene. Previous work in our laboratory has shown that introduction of human chromosome 11 into the highly metastatic breast carcinoma cell line MDA-MB-435 significantly reduces the metastatic potential of the hybrid clones [19]. Metastasis suppression was not observed with the control chromosome 6 microcell hybrids. This functional experimental evidence corroborated with primary breast tumor cytogenetic findings by also indicating that chromosome 11 harbors a metastasis-suppressor gene for human breast cancer.

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Abbreviations: LOH, loss of heterozygosity; TM4SF, transmembrane-4-super family; PCR, polymerase chain reaction.

To date, only a few metastasis-suppressor genes have been isolated from the human genome [20]. The *kai-1* gene, also known as CD82 (C33 antigen), maps to 11p11.2 and codes for a glycoprotein in the transmembrane 4 super family (TM4SF) of proteins [13,21–22]. Because it is operative in suppressing prostate cancer metastatic ability, *kai-1* is also an attractive candidate for a breast cancer metastasis-suppressor gene on chromosome 11. In addition, the gene for another TM4SF member, tapa-1 (CD81), also maps to chromosome 11, at band p15.5 [23]. Although tapa-1 has not been implicated in cancer suppression, it has been shown to induce cell-cell adhesion [24,25], a property often altered in metastatic cells [18]. The functions of these TM4SF surface proteins are largely unknown. Some associate with cell-surface receptors and are components of signaling complexes [26], whereas others may be important for maintenance of cell integrity, proliferation, and adhesion [27]. Therefore, alterations in expression levels of the kai-1 protein, the tapa-1 protein, or both could result in acquisition of invasive or metastatic ability.

To determine if either the kai-1 or tapa-1 proteins might control human breast metastatic ability, we examined their expression by western blot analysis in chromosome 11 microcell hybrids, MDA-MB-435 parental cells, and chromosome 6 controls. We also directly tested the role of the *kai-1* gene in our system by transfection of *kai-1* cDNA into both a mass population of MDA-MB-435 and a well-characterized subclone. Our data showed a positive correlation between increased kai-1 protein production and decreased metastatic ability among the chromosome 11 microcell hybrids. However, the *kai-1* transfectants were not significantly altered for metastatic ability. The transfectants expressed a more heavily glycosylated form of the protein than the microcell hybrids did, and there was a decrease in kai-1 protein in the primary tumors and metastatic lesions of the transfectants. Our results suggest that progression to metastasis in breast cancer may occur by a mechanism that either fails to check the degree of post-translational modification of kai-1, downregulates kai-1 protein production below some threshold level, or involves interactions with other proteins encoded by genes on chromosome 11.

MATERIALS AND METHODS

Cell Lines

Chromosome 11 and chromosome 6 microcell hybrids were generated as previously described by introducing single human chromosomes tagged with the neomycin-resistance gene into the highly metastatic human breast carcinoma cell line MDA-MB-435 [19]. Chromosome 11 microcell hybrid clones (neo11/435.A3, neo11/435.B1, neo11/435.D1, and neo11/435.E1) were derived from fusions using the mass population of MDA-MB-435 cells. Chromosome 6 microcell hybrid clones (neo6/435.7.A1, neo6/435.7.C1, neo6/435.7.E2, and neo6/435.7.GI) were derived from fusions using a subclone (MDA-MB-435.sub7) of the mass population. Transfected cDNA clones, designated Kai1/435.Pick1, Kai1/435.Pick 2, Kai1/435.Pick3, and Kai1/435.Pick 4, were generated by lipofectin-mediated transfection of the MDA-MB-435 cells with the supercoiled 8.2-kb plasmid pCMV-kai1 [13]. Transfectants were also made from a subclone of MDA-MB-435 (MDA-MB-435.sub1), and these were designated Kai1/435.sub1.Pick1, Kai1/435.sub1.Pick3, and kai1/435.sub1.Pick4. The expression vector alone was used to generate neo-transfected control clones, designated neo/435.A8, neo/435.B8, neo/435.C8, neo/435.D8, and neo/435.E8. All cell lines were maintained in RPMI and 10% fetal bovine serum, and microcell hybrids and transfectant clones were also supplemented with 600 μg/mL G418.

Western Blot Analysis

Cells were harvested from near-confluent T-75 flasks and lysed according to a previously described protocol [21]. Proteins were separated on 12.5% nonreducing sodium dodecyl sulphate–polyacrylamide gels containing 20–40 μg of nonreduced sample per lane. The protein was transferred to membranes (Immobilon-P; Millipore Corp., Bedford, MA) and incubated overnight in phosphate-buffered saline, 0.1% Tween-20, and 10% dry milk before addition of antibody for the TM4SF proteins. C33 (kai-1) and CD81 (tapa-1) antibodies were detected by using standard enhanced chemiluminescence techniques (ECL; Amersham Corp., Arlington Heights, IL). The C33 and TAPA1 monoclonal antibodies were kind gifts from Osamu Yoshie and Shoshana Levy, respectively [22,28].

Because the β-actin antibody does not hybridize to a blot run under nonreducing conditions, loading-control analysis was accomplished by simultaneously running a second gel loaded with equivalent amounts of dithiothreitol-reduced sample. Otherwise, treatment of the reduced gel and blot was identical to that of the kai-1 western blot. The protein levels were quantified by densitometric analysis with the NIH Image 1.55 program.

Polymerase Chain Reaction Analysis

The presence of full-length *kai-1* cDNA in the transfected cell lines was confirmed by polymerase chain reaction (PCR). Genomic DNA was amplified by using previously described primers [13] for the cDNA insert, yielding an amplicon of approximately 1 kb. The PCR solutions contained 400 ng of genomic DNA from parental cells and transfectants, standard buffer components, and 1.0 mM $MgCl_2$. The reaction conditions (50 μL) consisted of 30 cycles at 94°C for 1 min; 62°C for 2 min; and 72°C, for 2 min. The PCR

products were visualized in ethidium bromide–stained 2% agarose gels.

Spontaneous Metastasis Assay

Cells (1×10^6) were injected into the subaxillary mammary fat pads of 3- to 5-wk-old female, non-ovarectomized, athymic mice (Harlan Sprague Dawley, Madison, WI). The animals were killed about 3 mo after inoculation when their tumors were 1.5–2.0 cm in diameter or when the mice were moribund. In some mice, the primary tumors were surgically removed at about 60 d, but the animals were not killed until 90 d after inoculation to allow existing lung metastases to enlarge. Visible lung metastases were counted in Bouin's-fixed tissues, as previously described [29].

Cellular Morphology Analysis

All cell lines (1×10^6 cells) were plated into T-25 flasks (Corning, Corning, NY) and photographed 3 d later. Cell lines were visualized with an inverted microscope (Carl Zeiss, Inc., Thornwood, NJ) at 200×.

RESULTS

Characterization of kai-1 Expression in Parental and Microcell Hybrid Cell Lines by Western Blot Analysis

To determine the kai-1 protein levels of the metastatic MDA-MB-435 breast cancer cells and the four metastasis-suppressed chromosome 11 microcell hybrids, lysates were prepared from each cell line for western blot analysis with C33 antibody. As shown in Figure 1, almost no protein was expressed in parental MDA-MB-435 and MDA-MB-435.sub1 cells (lanes 1 and 2). However, each metastasis-suppressed neo11/435 hybrid (lanes 3–6) showed an increased level of kai-1 protein (Table 1) [19]. As was previously shown with immunoprecipitation experiments, the normal size of the kai-1 protein varies from 40 to 75 kD; the protein produces one to three major bands corresponding to its various N-glycosylation states [22]. kai-1 in the chromosome 11 microcell hybrids fell within this range (46–56 kD) and had the normal two- or three-band pattern. One of the hybrids, neo11/435.E1, expressed amounts of kai-1 similar to those of a prostate microcell hybrid line (AT6-11-1), which is also suppressed for metastasis [13]. These data showed that introducing chromosome 11 into the metastatic breast cell line resulted in increased kai-1 protein expression that correlated with metastasis suppression (Figure 1 and Table 1).

To determine if the increase in kai-1 expression was specific to chromosome 11 microcell hybrids, we also examined the four chromosome 6 cell lines that remained metastatic [19]. Figure 2 shows a western blot comparing the amount of kai-1 protein in three parental cell lines with chromosome 6 and chromosome 11 microcell hybrids and *neo*-transfectant controls. Again, the four lanes with lysate from the metastasis-suppressed cell lines (neo11/435.A3, neo11/435.B1, neo11/435.D1, and neo11/435.E1) had the highest levels of the kai-1 protein. Three of four chromosome 6 microcell hybrids (lanes 4–7) showed kai-1 levels that were not significantly greater than those of the parental cells and *neo* transfectants. Interestingly, neo6/435.7.G1 (lane 7) showed some increase in kai-1 expression. We previously showed that whereas these cells are not significantly suppressed for metastasis, they are the least aggressive of the four chromosome 6 hybrid cell lines [19]. The kai-1 blot was stripped and reprobed for tapa-1 expression, revealing only a slight increase in the levels of this protein among the metastasis-suppressed cell lines. Hybridization of the blot with

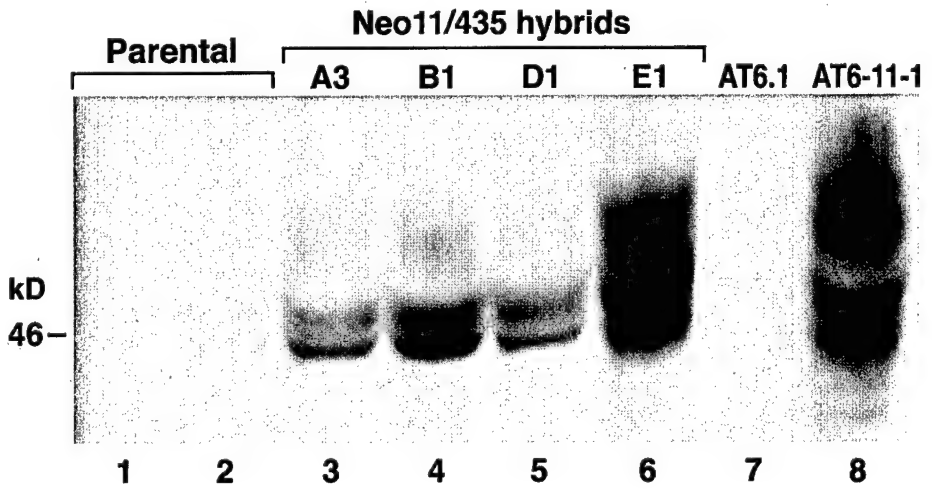

Figure 1. western blot analysis of kai-1 protein levels for MDA-MB-435 and MDA-MB-435.sub1 parental cells (lanes 1 and 2, respectively), chromosome 11 microcell hybrids (lanes 3–6), and negative (AT6.1) and positive (AT6-11-1) control cell lines. (AT6-11-1 is a metastasis-suppressed chromosome 11 microcell hybrid of the highly metastatic AT6.1 prostate cell line.)

Table 1. Metastasis Analysis of Transfectants and Microcell Hybrids

Cell Line*	Mean no. of Metastases	Range of Metastases	Incidence of Metastases	
			Ratio†	Percentage
MDA-MB-435				
Parental cells	16.1	0–130	28/32	87.5
neo11/435.A3	0.9	0–8	3/15	20.0
neo11/435.B1	1.7	0–37	2/26	7.7
neo11/435.D1	0.8	0–6	6/16	37.5
neo11/435.E2	1.5	0–24	3/17	17.6
neo/435.A8‡	5.2	0–35	9/15	60.0
neo/435.B8‡	7.0	0–37	12/14	85.7
neo/435.C8‡	1.0	0–3	9/15	60.0
neo/435.D8‡	1.5	0–7	9/15	60.0
neo/435.E8‡	2.4	0–9	10/15	66.6
Kai/435.Pick1	2.7	0–22	16/23	69.6
Kai/435.Pick2	7.1	0–23	14/19	73.7
Kai/435.Pick3	8.0	0–67	16/22	72.2

*At least three single-cell clones of each group were expanded for inoculation into the mammary fat pads of nude mice with one inoculation site per animal.
†Number of mice with lung metastases/total number of mice inoculated. All metastases were counted at least 92 d after inoculation, except for the *neo*-transfectant controls, which had to be killed early to prevent compromising animal welfare due to a large tumor burden. The data are results from at least two independent experiments.
‡Animals killed early (76d).

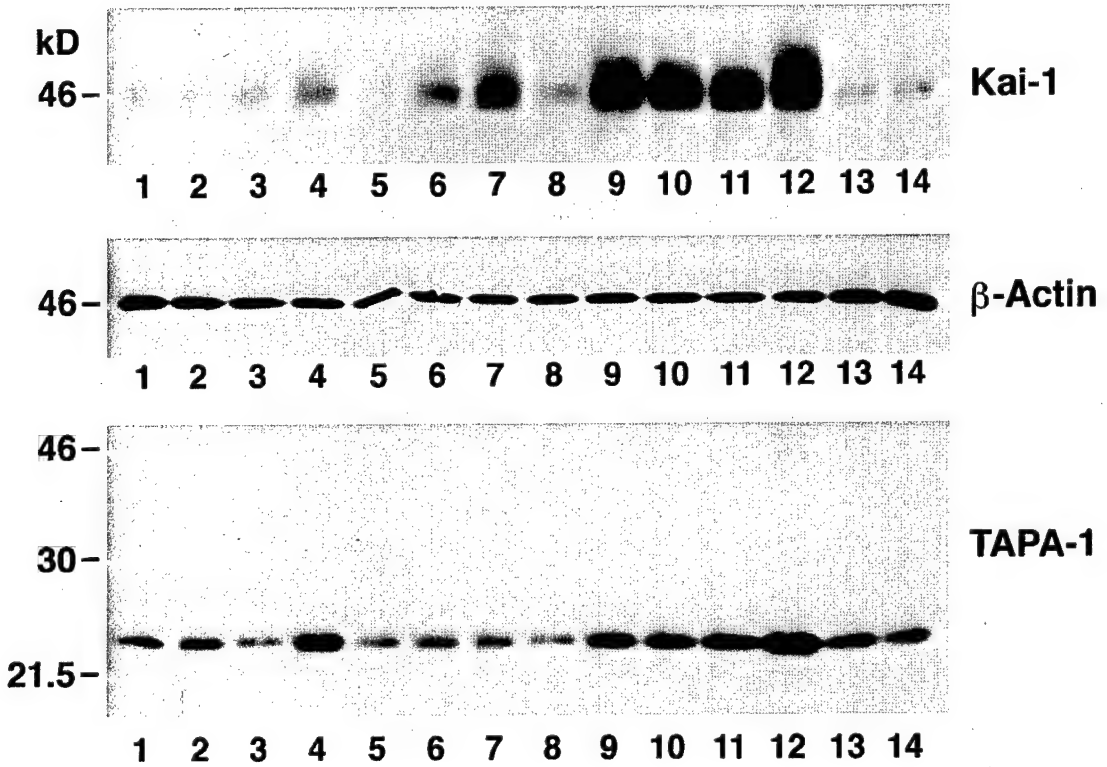

Figure 2. Relative amounts of kai-1 and tapa-1 proteins in the metastatic parental MDA-MB-435 mass population and two subclones (lanes 1–3, respectively), metastatic lines neo6/435.7.A1, neo6/435.7.C1, neo6/435.7.E2, and neo6/435.7.G1 (lanes 4–7, respectively); *neo*-transfectant controls (lanes 8–10); and metastasis-suppressed lines neo11/435.A3, neo11/435.B1, neo11/435.D1, and neo11/435.E1 (lanes 11–14, respectively). The lanes were loaded with 20 μg of protein, probed with a 1:50 dilution of C33 antibody, and then stripped and reprobed with a 1:1000 dilution of tapa-1 antibody. Protein size is given in kDa to the left of each blot. The western blot of β-actin antibody is a separate blot made from reduced samples and was used to assess equal lane loading.

β-actin antibody indicated that the lanes were evenly loaded. These data strongly indicate that kai-1 expression, and not tapa-1 expression, in the neo11/435 microcell hybrids correlates with the ability to suppress metastasis.

Transfection of the *kai-1* Gene into MDA-MB-435

To determine if the increase in kai-1 protein was responsible for the metastasis suppression seen in the chromosome 11 microcell hybrids, *kai-1* cDNA was transfected into the MDA-MB-435 cell line. Isolated colonies of presumed clonal origin, designated Kai1/435.Pick1, Kai1/435.Pick2, Kai1/435.Pick3, and Kai1/435.Pick4, resulted from transfection of the full-length *kai-1* cDNA into the mass population of MDA-MB-435 cells. Kai1/435.sub1.Pick 1, Kai1/435.sub1Pick3, and Kai1/435.sub.Pick4 clones resulted from transfection into the MDA-MB-435.1 subclone. PCR was used to verify that the insert was successfully transfected into each clone (Figure 3). Five of the seven *kai-1* transfectants (Figure 3, lanes 3–9) showed the expected 1-kb PCR product, which the parental cells (lanes 1 and 2) and the *neo*-transfected control cell lines (lanes 10–14) lacked. The *kai-1* cDNAs of Kai1/435.Pick 1 and Kai1/435.Pick 3 did not amplify to the same extent as that of other transfectant clones, and we presume that a full-length cDNA copy of the gene was not successfully introduced into these two transfectants.

To determine the level of kai-1 expression in the transfected clones, each clone was checked by western blotting. Figure 3 shows the level of expression in several *kai-1* transfectants after a short (15 s) enhanced chemiluminescence exposure. Although distinct bands were evident, several of the clones had protein in a wider size range (40–75 kDa) than the microcell hybrids did. This is suggestive of more extensive kai-1 protein post-translational modification, presumably heavier glycosylation. A longer exposure of this blot showed that Kai1/435.Pick1 and Kai1/435.Pick3 (Figure 3, lanes 1 and 3, respectively) express levels of the kai-1 protein that are increased over the parental cells (data not shown), however these two clones express the least amount of kai-1 compared with the other transfectants. This was expected, because PCR indicated that the full-length copy of *kai-1* might not have been present. Kai1/435.sub1.Pick1 and Kai1/435.sub1.Pick3 (Figure 3, lanes 5 and 7, respectively), had the highest levels of kai-1 protein. (The *kai-1* transfectants in lanes 6 and 8 had comparatively low levels of protein and were not further examined.) Three of the transfectants

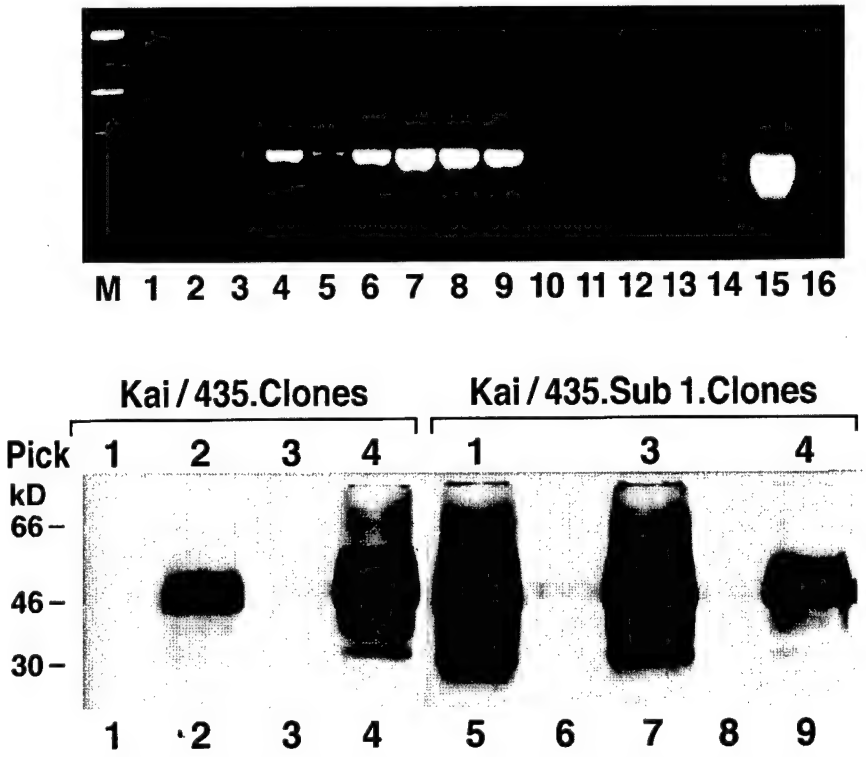

Figure 3. Upper panel: *kai-1* PCR of MDA-MB-435 and MDA-MB-435.sub1 parental cells (lanes 1 and 2, respectively), Kai1/435.Pick1, Kai1/435.Pick2, Kai1/435.Pick3, and Kai1/435.Pick4 (lanes 3–6, respectively), Kai1/435.sub1.Pick1, Kai1/435.sub1.Pick3, Kai1/435.sub1.Pick4) (lanes 7–9, respectively), *neo*-transfectants (neo/435.A8, neo/435.B8, neo/435.C8, neo/435.D8, and neo/435.E8) (lanes 10–14, respectively), supercoiled *kai-1* plasmid (lane 15), and a water blank (lane 16). The PCR products of about 1000 bp correspond to cDNA sequence present only in the *kai-1* transfectants and whole plasmid. M, marker. Lower panel: Western blot of the *kai-1*–transfected clones. Lanes 1–9 each contain 20 μg of total protein lysate from different *kai-1* transfectant clones. Seven of the nine clones were designated as Picks and expanded for inoculation into nude mice.

(lanes 4, 5, and 7) had considerable amounts of protein smaller than the expected 46 kDa. This may result from inefficient clearing of protein degradation products in cells that are overproducing the protein. These data show that, unlike our observations with the chromosome 11 microcell hybrids (Figure 1), the amount of kai-1 protein and the degree of posttranslational glycosylation vary greatly among transfectants. Furthermore, because the transfectants shown in Figure 3 expressed kai-1, which appeared to be more heavily modified than that of the microcell hybrids (Figure 1), the kai-1 expression from an introduced vector appeared to be qualitatively distinct from that seen after introduction of a whole, normal chromosome 11.

Characterization of In Vitro Morphology Differences

To determine if the variations in kai-1 expression and glycosylation affect cell phenotype, the cell size and shape in culture of each of the microcell hybrids and transfectants was compared. Because the TM4SF proteins are cell-surface molecules, the amount of kai-1 expression, the degree of glycosylation, or both could presumably affect cell-cell interactions. Figure 4 shows representative photographs of cell lines with lesser amounts of kai-1 (two left panels) next to cell lines with greater and more glycosylated amounts of the protein (two right panels). The neo11/435.D1 cells were more compact and more spindle-shaped than the neo11/435.E1 cells. Likewise, Kai1/435.sub1.Pick4 grew much more compactly in culture, and the cells were smaller than the Kai1/435.sub1.Pick3 cells. In general, for all cell lines, there were morphological differences in culture that correlated with the expression level and glycosylation pattern of the kai-1 protein. Furthermore, population doubling time tended to be higher in those cell lines that grew less compactly (data not shown). These data indicate that the level of kai-1 expression may influence in vitro phenotypic growth characteristics.

Metastasis Assay of Transfectant Clones

We next tested whether *kai-1* transfectants showed reduced metastatic potential. Transfectant clones were inoculated into the mammary fat pads of athymic nude mice and assayed for metastatic ability. Table 1 lists the metastasis data for the pa-

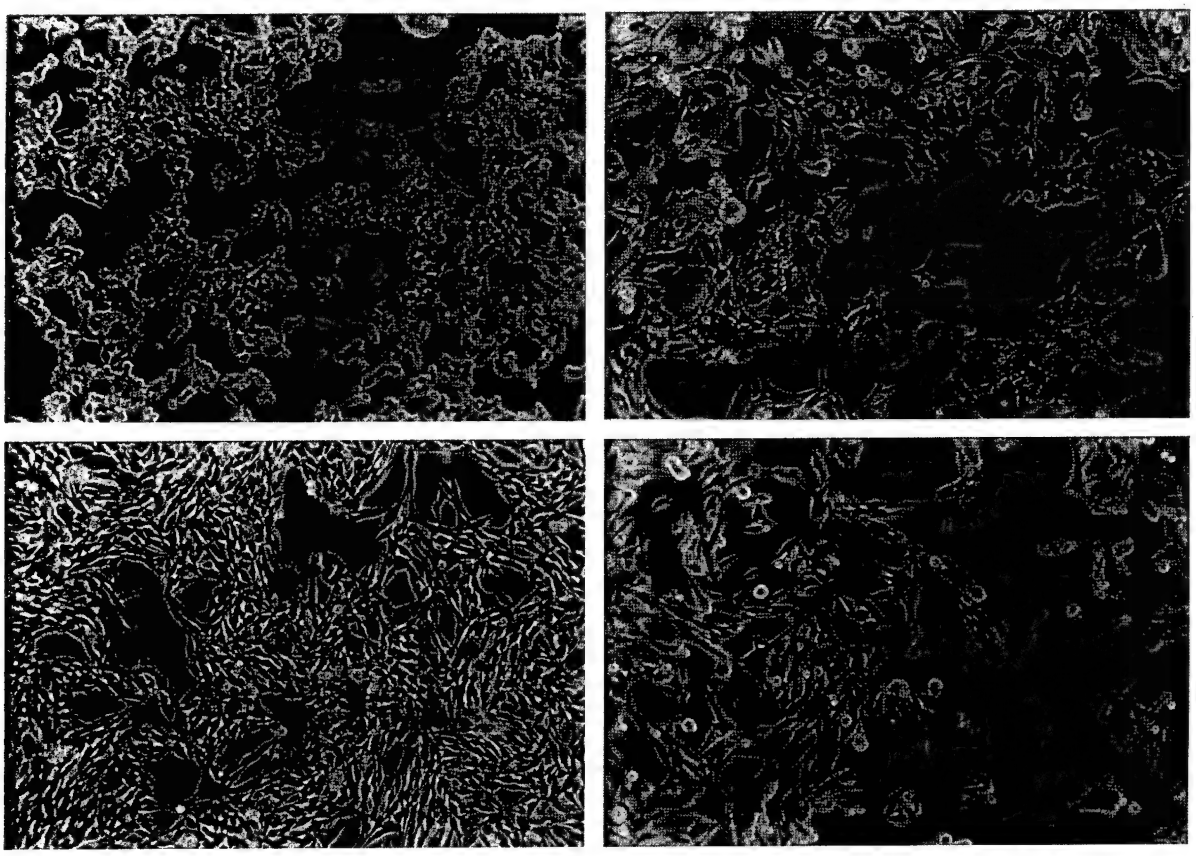

Figure 4. Photomicrographs (200×) of cells growing in culture: neo11/435.D1 (upper left), neo11/435.E1 (upper right), Kai1/435.sub1.Pick3 (lower left), and Kai1/435.sub1.Pick4 (lower right).

rental cell line, *kai-1* transfectants, *neo*-transfectant controls, and chromosome 11 microcell hybrids.

As with most solid tumors, inherent heterogeneity for metastatic potential existed within the MDA-MB-435 parent cell population. As seen with five clones transfected with the *neo* vector alone, the average number of metastases per lung varied over a large range, whereas the incidence of metastases was similar to that of the parental cell line (Table 1). Similar results were observed with the *kai-1* transfectants, indicating no differences between the control neoR-transfectants and the kai-1–producing transfectants. The only cells that produced significantly fewer lung metastases were from the chromosome 11 microcell hybrid group (Table 1). Introduction of *kai-1* into the Kai1/435.sub1 cell line had little effect on its metastatic potential (Table 2). This indicated that a gene other than the *kai-1* gene on chromosome 11 is important in controlling metastasis in the neo11/MDA-MB-435 model.

kai-1 Expression in Primary Tumors

There is one other possible explanation for the lack of significant metastasis suppression among the *kai-1* transfectants. Loss of the cDNA or failure to continue expressing kai-1 protein could explain the seeming disparity between the western blot analysis data and the lack of metastasis suppression in the *kai-1* transfectants. To test this possibility, locally growing tumors were analyzed for kai-1 protein expression and compared with inocula. Figure 5 shows a western blot of inoculated cells and locally growing tumors for three *kai-1* transfectants and the MDA-MB-435 parental cells. The blot showed a decrease in the amount of kai-1 protein in each transfectant primary tumor, ranging from 45% (Kai1/435.sub1.Pick1) to 22% (Kai1/435.Pick4) (Figure 5). Very little protein could be detected in the primary tumor or the metastatic lesions of the MDA-MB-435 cells, but the inoculated cells showed the same low level (Figure 1). We also observed a 50% decrease in the lung metastases of the Kai1/435.Pick 4 cell line. A comparable analysis of three inoculated neo11/435 hybrids (neo11/435.A3, neo11/435.B1, and neo11/435.E1) with matched locally growing tumor proteins (Figure 6) showed slightly decreased levels of kai-1 (75% in neo11/435.E1 to more than 500% for neo11/435.B1) for all hybrids. The neo11/435.B1 tumor reconstitute showed an unexplained dramatic increase in kai-1 expression over that of inocula, as did one of the neo11/435.D1 tumors in a preliminary experiment (data not shown). These data show that kai-1 expression in the transfectants was consistently reduced in vivo during tumor progression to metastasis, perhaps owing to the heavy glycosylation. The chromosome 11 microcell hybrids, which were metastasis suppressed, generally showed no such decrease in kai-1 expression in vivo. Taken together, the western blot, transfection, and in vivo expression data suggest that *kai-1* is a metastasis-suppressor gene on chromosome 11 for breast cancer, and metastasis is permitted only when protein levels fall below a threshold, when the protein is heavily modified post-translationally, or both.

DISCUSSION

A previous study showed that chromosome 11 harbors a strong metastasis-suppressor gene for the MDA-MB-435 cell line. Candidate breast cancer metastasis-suppressor genes on chromosome 11 are *kai-1* and *tapa-1*, which code for structurally related proteins, respectively mapped to 11p11.2 and 11p15.5. The *kai-1* gene is a particularly attractive candidate for several reasons. It is a known prostate cancer suppressor gene, and its expression in tumors was recently found to correlate with favorable outcome in non–small cell lung cancer patients [30]. Also, similar to our findings with breast cancer, *kai-1* expression is decreased in pancreatic metastases [31]. Both *kai-1* and *tapa-1* express proteins that are TM4SF family members and probably function in maintenance of cell integrity and adhesion. Also, markers surrounding the *tapa-1* locus show LOH in breast tumors [2], thus suggesting the presence of a suppressor gene in the region. Furthermore, both prostate and breast tissues are hormonally regulated; hence, loss of hormonal regulation could result from the loss of activity of a common suppressor gene.

In this study, we wanted to determine whether either *kai-1* or *tapa-1* was the operative metastasis-suppressor gene in our microcell hybrid cell lines. *tapa-1* appears to be a poor candidate, as western blot analysis revealed only a slight increase in the protein expression levels that correlated with metastatic potential. We presume that this increase is due to expression from three normal alleles. In contrast, western blots showed that kai-1 levels inversely correlated with metastatic ability in the microcell hy-

Table 2. Metastasis Analysis of MDA-MB-435.sub 1 Transfectants

Cell Line	Mean # of Metastases	Range of Metastases	Incidence of Metastases	
			Ratio*	Percentage
MDA-MB-435.sub1	5.8	2–15	4/4	100.0
Kai1/435.sub1.Pick1	3.0	0–7	3/4	75.0
Kai1/435.sub1.Pick3	7.5	2–15	4/4	100.0

*Number of mice with lung metastases/total number of mice inoculated. All metastases were counted at least 90 d after inoculation. Primary tumors were removed at 60 d to reduce tumor burden on the animals.

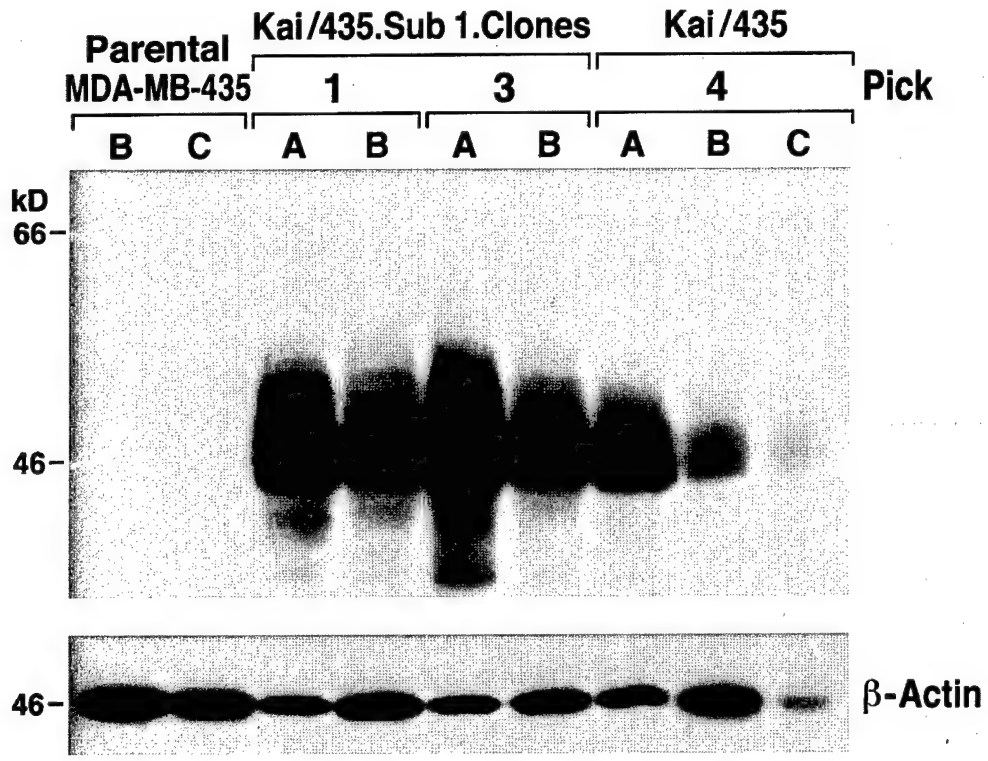

Figure 5. Western blot of matched sets of parental cells and three transfectant clones, showing kai-1 levels of inoculated cells (A), primary tumors (B), and lysates from metastatic lesions (C). Ten micrograms of protein was loaded in each lane. Equal loading control was assessed with a separate blot run identically and simultaneously with the kai-1 blot, except that the sample was reduced before loading.

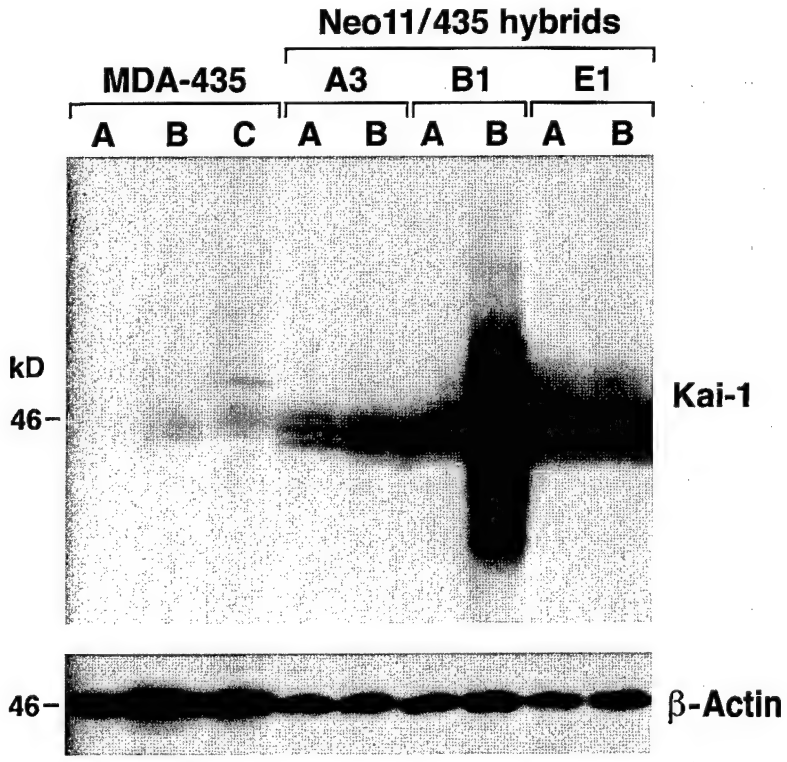

Figure 6. Western blot of matched sets of parental cells and three chromosome 11 microcell hybrids, showing kai-1 levels for inoculated cells (A), primary tumors (B), and lysates from metastatic lesions (C). Ten micrograms of protein was loaded in each lane.

brids. This result was consistent with the metastasis suppression resulting from transfer of normal human chromosome 11 and could be caused by an increase in kai-1 protein expression.

To test the importance of *kai-1* as a metastasis-suppressor gene in breast cancer, we transfected full-length *kai-1* cDNA under the control of a constitutive cytomegalovirus promoter into MDA-MB-435. In theory, if *kai-1* were the key gene controlling metastasis, the same strong suppression of metastasis seen in the chromosome 11 microcell hybrids should have been seen in the transfectants. In this study, we could not clearly establish an effect of *kai-1* expression on metastasis, because *kai-1* transfectants were not significantly suppressed for metastasis. However, there were two important differences between the protein in the transfectant clones and the chromosome 11 microcell hybrids. First, many of the transfectant clones produced more heavily modified kai-1 protein, the amount of which seemed to increase with decreased confluency in culture. This difference did correlate with metastatic ability among the transfectant clones themselves in that the most glycosylated clones tended to form the most metastatic lung lesions. Heavy glycosylation was not seen with the suppressed chromosome 11 microcell hybrids, perhaps because other genes alter posttranslational modification (e.g., fucosyltransferase on 11q21 [32]). Deglycosylation experiments could better clarify these data. Second, analysis of the metastatic lesions and primary tumors by western blotting revealed that kai-1 levels had been reduced in vivo for all transfectant cell lines. We could not eliminate the possibility that kai-1 levels dropped below a threshold level required for metastasis suppression. Examining *kai-1* gene expression under the control of a stronger or inducible promoter may allow us to answer this question.

Why do metastatic MDA-MB-435 cells express a reduced level of kai-1 protein? There may be loss of some trans-activating factor or factors necessary for expression, or expression could be altered by DNA modification of the *kai-1* gene itself. In fact, we have preliminary evidence that endogenous *kai-1* genes in metastatic MDA-MB-435 could be silenced because of methylation (Phillips KK, Weissman BE, unpublished observations). These data support the observations of Dong et al. [33], which suggest that methylation could be silencing *kai-1* in prostate tumors. Demethylation experiments with 5-azacytidine could help clarify these data.

In summary, our results indicated the *kai-1* metastasis-suppressor gene may contribute to the progression of breast cancer in humans. Our results suggest two possible mechanisms: reduction of expression in vivo and posttranslational modification, which affects normal function. Furthermore, we cannot rule out the possibility that kai-1 acts in concert with other genes on chromosome 11 to control breast cancer malignancy. The modest decreases in metastasis observed in the transfectants support this theory. More thorough analyses of the mechanisms mediating kai-1 protein function will help to elucidate these possibilities.

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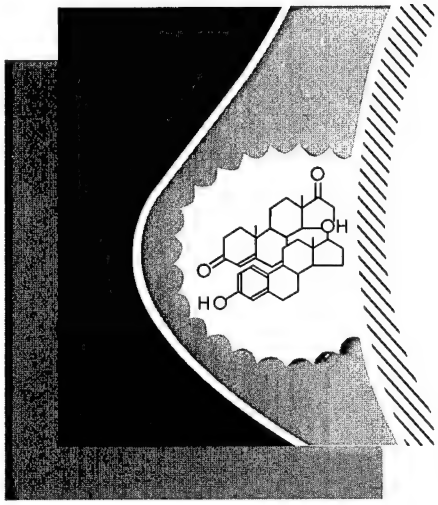

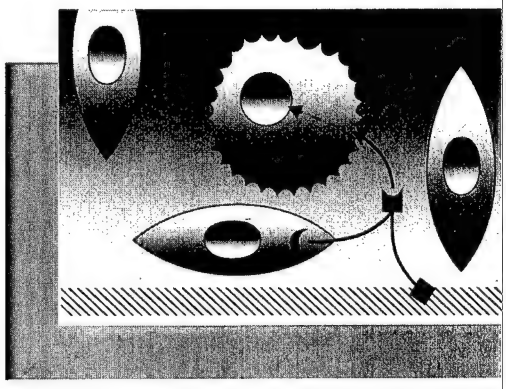

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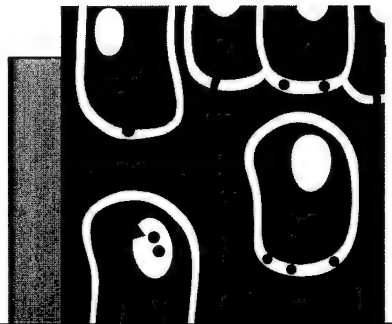

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Genetic and epigenetic regulation of human breast cancer progression and metastasis

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Introduction

Breast cancer is the most common malignancy and a major cause of cancer-related deaths among women in the United States and Western Europe (American Cancer Society 1998, Wingo *et al.* 1998). Most women succumb to breast cancer if their tumors metastasize but cures are more likely if the cancers remain localized (Harris *et al.* 1992*a*,*b*,*c*, Walker *et al.* 1997). Thus, a greater understanding of the metastatic process in human breast cancer should translate into substantial improvements in therapeutic outcome for breast cancer patients. Towards that end, we will review and summarize the literature about, and begin to develop a working model for, the genetics of human breast cancer metastasis. There have been great strides in recent years with regard to our overall understanding of metastasis. Yet our apparently straightforward objective — to define cause-effect relationships for genes in breast cancer — was difficult because of four issues. First, many reports fail to distinguish between oncogenesis and progression or invasion and metastasis when reporting data. Secondly, there is a failure, by some, to recognize that breast cancer is not a single disease, but a collection of diseases. This is particularly apparent in the genetics literature. Thirdly, it is difficult to evaluate the relative importance of correlative data, particularly as they relate to mechanistic control of steps in the metastatic cascade. Fourthly, there is a tremendous noise-to-signal ratio for genetics of late-stage, metastatic breast cancers resulting from genotypic instability, phenotypic drift and tumor heterogeneity.

There are several assertions in the literature claiming a role for genes in controlling progression and/or metastasis of breast cancer. Out-of-hand dismissal of some of those claims was possible because the studies lacked necessary controls. For other genes, the data were more preliminary or correlative and, for an extremely small number of genes, functional data demonstrating regulation of breast cancer metastasis were available. The text of this review will focus on the latter; however, we decided that the utility of this article would be maximized if we summarized the known role(s) of individual breast cancer-associated genes, clearly discriminating the genes that regulate oncogenesis from those that control metastasis. The most effective method to accomplish this goal was to create tables that summarize the references providing evidence for a particular role(s) of genes in human breast cancer. Table 1 is designed to be used as a resource. Putative role(s) of individual genes in breast cancer are separated into two categories — oncogenesis and progression/metastasis — where key references are given to substantiate/refute a role. Although we attempted to be thorough and inclusive, the extensive historical literature combined with the rapidly evolving breast cancer genetics field limit the completeness of this review. We apologize to those whose work was not included because of space considerations or whose papers were inadvertently omitted. However, we hope that this review fulfils our fourfold objective: (1) to highlight the genes for which roles in late-stage human breast cancer and/or metastasis have been functionally demonstrated; (2) to distinguish those genes from the more numerous oncogenic or tumor suppressors involved in breast cancer; (3) to evaluate the literature in order to identify needs for the field of breast cancer metastasis research to move to the next level; and (4) to propose a working model for the genetics of human breast cancer progression, focusing on the genes that have demonstrable metastasis-regulatory activity.

Breast cancer is a collection of diseases

Invasive breast cancers are an histologically and biochemically heterogeneous set of diseases. Lesions are typically categorized on the basis of histological appearance, resembling either ductal or lobular components of the healthy breast. Most studies suggest that the majority of tumors arise in the terminal ductal unit of the breast, perhaps in a single type of 'target' cell (Goehring & Morabia 1997, Russo & Russo 1997). By far

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Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer.

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
APC / FAP	5q21	Regulate β-catenin; cytoskeletal organization	LOH	(Thompson *et al.* 1993)	
ATM ataxia-telangiectasia	11q22-q23	DNA repair	LOH, mutation	(Athma *et al.* 1996; Carter *et al.* 1994; Cortessis *et al.* 1993; Ferti-Passantonopoulou *et al.* 1991; Hampton *et al.* 1994; Kerangueven *et al.* 1997; Tomlinson *et al.* 1995; Vorechovsky *et al.* 1996)	
α-catenin	5q31	Cytoplasmic component of E-cadherin; cytoskeletal organization	Reduced expression: (Glukhova *et al.* 1995; Rimm *et al.* 1995) Mutation: (Rimm *et al.* 1995)		Invasion: (Glukhova *et al.* 1995; Rimm *et al.* 1995)
bcl-2	18q21	Apoptosis; interacts with c-myc	Overexpression, amplification		Progression: (Olopade *et al.* 1997; Silvestrini *et al.* 1994; Zschiesche *et al.* 1997)
BrCa1	17q21		LOH, mutation	(Casey 1997; Dickson & Lippman 1995; Holt *et al.* 1996; Rao *et al.* 1996)	
		DNA repair, genome stability		(Scully *et al.* 1997)	
		Cell cycle		(Chen *et al.* 1996; Futreal *et al.* 1994; Larson *et al.* 1997; Miki *et al.* 1994; Somasundaram *et al.* 1997; Wang *et al.* 1997a)	
		Differentiation		(Boyd *et al.* 1995; Goldman *et al.* 1997; Hakem *et al.* 1996; Ludwig *et al.* 1997; Marquis *et al.* 1995)	
		Apoptosis		(Shao *et al.* 1996)	

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
Thymosin β15		Cytoskeletal organisation, motility	Increased mRNA expression		Progression (Bao *et al.* 1998)
BrCa2	13q12-q13		LOH, mutation	(Casey 1997; Cleton-Jansen *et al.* 1995; Collins *et al.* 1995; Wooster *et al.* 1995)	
		DNA repair, genome stability		(Patel *et al.* 1998; Sharan *et al.* 1997)	
		Differentiation		(Ludwig *et al.* 1997)	
		Cell cycle		(Wang *et al.* 1997)	
BrCa3	8p12-p22	DNA repair	LOH	(Casey 1997; Hoekstra 1997; Lavin & Shiloh 1997; Meyn 1995; Seitz *et al.* 1997)	
Brush-1	13q12-q13			(Schott *et al.* 1994)	
Cathepsin D	11p15-pter	Proteinase	Overexpression	(Westley & May 1996)	Progression/invasion: (Garcia *et al.* 1996; Johnson *et al.* 1993; Lah *et al.* 1995; Rochefort *et al.* 1990*a,b*; Tedone *et al.* 1997)
CD31 (PECAM)	17q23	Angiogenesis (marker)	Increased expression in stromal component		Progression/angiogenesis/invasion: (Charpin *et al.* 1995; Fox *et al.* 1997; Martin *et al.* 1997)
CD44	11p13	Adhesion	Amplification, Overexpression		Progression/invasion/metastasis: (Herrlich *et al.* 1993; Zöller & Kaufmann 1994)
					Progression/invasion/metastasis: (Hofmann *et al.* 1991; Joensuu *et al.* 1993)

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
c-erb-B2	17q12	Growth factor receptor Tyrosine kinase	Amplification: (28-35%) (Slamon *et al.* 1987; Spandidos *et al.* 1989; Zhou *et al.* 1989) Overexpression: (Anbazhagan *et al.* 1991; Gullick *et al.* 1991; Lovekin *et al.* 1991; O'Reilly *et al.* 1991)	Conflicting/controversial data. Studies are at odds, no consistent or defining studies	Stage/progression: (Hubbard *et al.* 1994; Liu *et al.* 1992; Press *et al.* 1994; Slamon *et al.* 1987, 1989) Control of metastasis or invasion: (Giunciuglio *et al.* 1995; Tan *et al.* 1997; Tavassoli *et al.* 1989; Yu & Hamada 1992; Yusa *et al.* 1990)
c-myc	8q24	Transcription: (Bonilla *et al.* 1988; Edwards *et al.* 1988) Growth, differentiation: (Evan *et al.* 1992) Apoptosis: (Chernova *et al.* 1998; Packham *et al.* 1996; Ryan & Birnie 1996; Wagner *et al.* 1994)	Amplification, overexpression, mutation	(Amundadottir *et al.* 1996*a*; Berns *et al.* 1992*a,b*; Dickson & Lippman 1995; Escot *et al.* 1986; Guerin *et al.* 1988; Kozbor & Croce 1984; Nass & Dickson 1997; Watson *et al.* 1996; Wong & Murphy 1991)	Progression: (Guerin *et al.* 1988; Tavassoli *et al.* 1989; Watson *et al.* 1993, 1996)
Cyclin D1	11q13	Cell cycle	Amplification, overexpression, mutation	Amplification: (Buckley *et al.* 1993; Courjal *et al.* 1996; Dickson *et al.* 1995; Gillett *et al.* 1994; Han *et al.* 1995; Peters 1994, 1995) Mutated: (Lebwohl *et al.* 1994) Overexpression: (Bartkova *et al.* 1994, 1995) In precursor lesion (DCIS to infiltrating ductal Ca): (Steeg *et al.* 1996; Weinstat-Saslow *et al.* 1995)	
Cyclin E	ND	Cell cycle	Overexpression	(Bortner & Rosenberg 1997; Gray-Bablin *et al.* 1996)	Progression: (Keyomarsi *et al.* 1994; Said & Medina 1995)

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
DCC	18q21		LOH	(Thompson *et al.* 1993)	
E-cadherin	16q22.1	Adhesion - homotypic	Reduced expression: (Palacios *et al.* 1995)	(Hirai *et al.* 1998; Lochter *et al.* 1997a)	Invasion/metastasis: (Guriec *et al.* 1996; Jones *et al.* 1996; Lipponen *et al.* 1994; Mbalaviele *et al.* 1996; Oka *et al.* 1993; Palacios *et al.* 1995; Siitonen *et al.* 1996; Berx *et al.* 1995; Perl *et al.* 1998; Rimm *et al.* 1995)
ERβ	6q24-q27	Hormone receptor, transcription	Mutation, loss of expression LOH	(Andersen *et al.* 1994)	Tumor progression: (Estes *et al.* 1987; Graham *et al.* 1990; Leygue *et al.* 1996*b*; Mackay *et al.* 1988; Magdelénat *et al.* 1994; Scott *et al.* 1991; Sheikh *et al.* 1994; Thompson *et al.* 1992) Invasion: (Garcia *et al.* 1992; Hoelting *et al.* 1995; Sheikh *et al.* 1994) Metastasis: (Fuqua *et al.* 1991*a*; Garcia *et al.* 1992)
ERβ	14q22-24	Hormone receptor, transcription	Mutation	(Dotzlaw *et al.* 1997; Enmark *et al.* 1997; Kuiper *et al.* 1996; Leygue *et al.* 1996*a*; Vladusic *et al.* 1998)	
ETS-2		Transcription	Overexpression		Invasion: (Sapi *et al.* 1998)
FGF family	Multiple	Growth factors, angiogenesis	Amplification, overexpression	(McLeskey *et al.* 1996; Payson *et al.* 1996; Penault-Llorca *et al.* 1995; Relf *et al.* 1997)	Progression: (Souttou *et al.* 1996) Metastasis: (Kern *et al.* 1994; McLeskey *et al.* 1993)

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis*
FHIT	3p14.2		LOH, mutation	(Mau *et al.* 1996; Negrini *et al.* 1996; Panagopoulos *et al.* 1996)	
		Fragile histidine triad; genomic stabilty		(Barnes *et al.* 1996; Huebner *et al.* 1997)	
		Hormone receptor, transcription		(Martin *et al.* 1993; Tonetti & Jordan 1997)	
IGF2R (mannose 6-phosphate receptor)	6q26-q27		Overexpression		Progression: (Chappell *et al.* 1997)
IL-1β	2q13	Cytokine	Increased expression		Progression: (Jin *et al.* 1997)
IL-8	4q13	Cytokine	Increased expression		Progression/angiogenesis: (Green *et al.* 1997)
int-1	12q13		Amplification	(Meyers *et al.* 1990)	
int-2/FGF-3	11q13	Growth factor	Amplification, overexpression	(Huebner *et al.* 1988; Liscia *et al.* 1989)	
KAI-1 (CD82)	11p11.2	Adhesion	Decreased expression		Progression: (Yang *et al.* 1997) Transfection/metastasis: (Phillips *et al.* 1998)
KiSS-1	1q32	Signal transduction	Decreased expression		Transfection/metastasis: (Lee & Welch 1997*b*)
Laminin-5	1q	Adhesion, invasion	Overexpression		Invasion: (Pyke *et al.* 1995)
mdm-2	12q13-q14	Inhibit TP53	Overexpression	(Jiang *et al.* 1997)	Progression: (Jiang *et al.* 1997)
MMPs / TIMPs	Multiple	Invasion		(Lochter *et al.* 1997*a*,*b*)	Progression: (Tryggvason *et al.* 1993) Experimental models: (Polette *et al.* 1997; Stonelake *et al.* 1997; Ueno *et al.* 1997; Wang *et al.* 1997)
		Angiogenesis		(Thorgeirsson *et al.* 1996)	

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
MnSOD (SOD2)	6q25	Reduce oxygen radicals	Decreased expression	(Li *et al.* 1995)	
MRP-1/CD9	12p13	Differentiation; motility	Loss of expression		Progression: (Miyake *et al.* 1995, 1996)
NFκB	4q24	Transcription	Overexpression	(Sovak *et al.* 1997)	
NME1 Nm23-H1	17q21.3	NDP kinase? Some find that NDPK activity is not associated with metastasis suppression (MacDonald *et al.* 1993)	Decreased expression, mutation		Lymph node status: (Barnes *et al.* 1991; Bevilacqua *et al.* 1989; Freije *et al.* 1996; Hennessy *et al.* 1991; Royds *et al.* 1993; Steeg *et al.* 1993; Tokunaga *et al.* 1993; Toulas *et al.* 1996) Histologic grade: (Hirayama *et al.* 1991; Yamashita *et al.* 1993) No correlation: (Goodall *et al.* 1994; Sastre-Garau *et al.* 1992; Sawan *et al.* 1994) Transfection/metastasis: (Fukuda *et al.* 1996; Leone *et al.* 1993)
		Growth		(Cipollini *et al.* 1997)	
NME2 Nm23-H2	17q	NDP kinase			Transfection/metastasis: (Fukuda *et al.* 1996; Kraeft *et al.* 1996) Transfection: (Postel *et al.* 1993) No suppression: (Tokunaga *et al.* 1993)
		c-myc transcription			
Nm23-DR	ND	Differentiation, apoptosis			
Nm23-H4	16p13	NDP kinase			

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
p16/p15/p19ARF	9p21	Cell cycle	Mutation, LOH: (Haber 1997)	(Brenner & Aldaz 1995; Geradts & Wilson 1996; Herman *et al.* 1995; Xu *et al.* 1994; Zariwala *et al.* 1996)	
$p21^{WAF1/CIP1/mda6/Sdi1}$	6p21	Cell cycle	Overexpression: (Lukas *et al.* 1997) Decreased expression: (Jiang *et al.* 1997)	(Lukas *et al.* 1997; Rey *et al.* 1998)	Progression: (Jiang *et al.* 1997)
p53 (TP53)	17p13.1		LOH, mutation, mutant overexpression: (Bennett *et al.* 1992; Gusterson *et al.* 1991)	(Bartek *et al.* 1990; Bukholm *et al.* 1997; Davidoff *et al.* 1991; Eyfjörd *et al.* 1995; Gusterson *et al.* 1991; Harris 1992; Hartmann *et al.* 1997; Horak *et al.* 1991; Jerry *et al.* 1993; Poller *et al.* 1992)	Progression: (Allred *et al.* 1993; Anbazhagan *et al.* 1991; Barnes *et al.* 1993; Casey *et al.* 1993; Chen *et al.* 1994; Gullick *et al.* 1991; Lovekin *et al.* 1991; Mazars *et al.* 1992; O'Reilly *et al.* 1991; Poller *et al.* 1992; Thor *et al.* 1992)
		Transcription		(Harris 1996; Levine 1997; Wang & Harris 1997)	
		Genome stability		(Levine 1997; Tlsty *et al.* 1993; Wynford-Thomas 1997)	
PR	11q13	Hormone receptor, transcription; marker for estrogen response	Decreased expression, mutation, LOH		Progression: (Ali *et al.* 1987; Fuqua *et al.* 1991*b*; Horwitz *et al.* 1982; Magdelénat *et al.* 1994; McGuire *et al.* 1986; Tomlinson *et al.* 1996)
PKCα	17q22-q23.2	Signal transduction			Invasion/metastasis: (Ways *et al.* 1995)
PKCδ		Signal transduction	Overexpression, activation	(Jaken *et al.* 1997; Kiley *et al.* 1996)	Transfection/metastasis: (Jaken *et al.* 1997; Kiley *et al.* 1996, 1998)
Mammaglobin	11q13	Steroid binding?	Overexpression, amplification	(Watson & Fleming 1996)	

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in: oncogenesis	clinical stage/grade, tumor progression or metastasis*
MMAC1/PTEN	10q23	Tyrosine phosphatase	LOH, mutation, decreased expression	Low importance: (Chen *et al.* 1998)	Progression: (Dahia *et al.* 1997; Li *et al.* 1997; Liaw *et al.* 1997; Lynch *et al.* 1997; Nelen *et al.* 1997; Okami *et al.* 1998; Rasheed *et al.* 1997; Rhei *et al.* 1997; Sakurada *et al.* 1997; Steck *et al.* 1997; Teng *et al.* 1997)
Ras	11p15	Signal transduction	Overexpression: (Spandidos *et al.* 1989; Thor *et al.* 1986) Mutations but rare: (Rochlitz *et al.* 1989; Thor *et al.* 1986) LOH: (Theillet *et al.* 1986)	(Thor *et al.* 1986)	Invasion (data controversial and contradictory): (Lundy *et al.* 1986; Spandidos *et al.* 1989)
Raf-1	3p25	Signal transduction	Overexpression (measured in cell lines only)		Progression: (Callans *et al.* 1995)
Rb1	13q14	Cell cycle	LOH, mutation	(Picksley & Lane 1994; Riley *et al.* 1994; Sherr 1994; Wang *et al.* 1994; Cox *et al.* 1994; Lundberg *et al.* 1987; Shackney & Shankey 1997; Spandidos *et al.* 1989; T'Ang *et al.* 1988; Zhou *et al.* 1989)	Progression: (Borg *et al.* 1992*a*; Varley *et al.* 1989)
Telomerase		Maintain telomere length	Increased activity		Progression: (Hoos *et al.* 1998)
TSP-1	15q15-q21		LOH, mutation, decreased expression, truncation	(Weinstat-Saslow *et al.* 1994; Zabrenetzky *et al.* 1994; Zajchowski *et al.* 1990)	Progression: (Walz 1992)
		Inhibit angiogenesis		(Castle *et al.* 1997; Dameron *et al.* 1994*a*,*b*; Volpert *et al.* 1995; Weinstat-Saslow *et al.* 1994)	Transfection/metastasis: (Weinstat-Saslow *et al.* 1994)
		Induce apoptosis		(Guo *et al.* 1997)	

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis*
			Increased expression		Pro-invasion: (Albo *et al.* 1997; Arnoletti *et al.* 1995; Tuszynski *et al.* 1987*a*; Wang *et al.* 1996) Pro-adhesion: (Incardona *et al.* 1995; Pratt *et al.* 1989; Tuszynski *et al.* 1987*b*) Anti-metastatic: (Zabrenetzky *et al.* 1994) Conflicting data (no correlation): (Bertin *et al.* 1997)
TGF-α	2p11-p13	Growth factor; synergistically induces mammary tumors with c-myc transgenic animals	Increased expression	Experimental systems: (Amundadottir *et al.* 1996*b*)	
TGF-β1	19q	Growth factor; can promote VEGF, or MMP expression	Increased protein expression, mutation	(Park *et al.* 1997) Growth inhibitor: (Arteaga *et al.* 1996; Butta *et al.* 1992; Mazars *et al.* 1995; Sun *et al.* 1994)	(Note: conflicting data that TGF-β1 inhibits or promotes progression) Increased invasiveness: (Hildenbrand *et al.* 1998; Oft *et al.* 1996; Welch *et al.* 1989) Progression: (Cardillo *et al.* 1997) Possible role in metastasis: (Walker *et al.* 1994)
TIMP-1	Xp11.23-p11.4	Inhibitor of MMPs	Increased expression	(Li *et al.* 1994)	Progression/invasion: (Yoshiji *et al.* 1996*b*)
TIMP-2	17q	Inhibitor of MMPs	Increased expression		Progression/invasion: (Visscher *et al.* 1994)
uPA / tPA PAI-1 / PAI-2	Various	Invasion	Increased expression (proteinases) Decreased expression (inhibitors)		Progression: (Duffy *et al.* 1996; Foekens *et al.* 1995; Ishikawa *et al.* 1996; Sappino *et al.* 1987)

Table 1 Genes reportedly involved in oncogenesis and/or progression of human breast cancer. (Continued)

Gene	Map location	Presumptive mechanism(s) of action	Identified aberrations in clinical samples or cell cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis*
VEGF	6p12-p21.3	Angiogenesis	Overexpression		Progression: (Anan *et al.* 1996; Guidi *et al.* 1997; Kern & Lippman 1996; Yoshiji *et al.* 1996*a*, 1997)
VHL	3p25-p26	Cell cycle; inhibits VEGF mRNA accumulation; binds to elongin	Mutations	(Beroud *et al.* 1998)	
WNT	Wnt14 1 Wnt13 1p13 Wnt15 17q21 Wnt3 17q21 Wnt5a 3p14-p21 Wnt10b 12q13	Most data are for murine tumors, but possible correlations exist in human breast carcinomas		Wnt-2 (Dale *et al.* 1996) Wnt14 and Wnt15 (Bergstein *et al.* 1997) Wnt10b (Bui *et al.* 1997)	

*Progression indicates only that correlations have been seen in clinical and/or experimental systems corresponding with advanced stage or grade. Attributes of later stages of progression for which specific data are correlated are noted. Table 1 contains some data from other models, particularly with regard to mechanism of action. However, most of the data presented are from breast or mammary tumors. LOH, loss of heterozygosity; MMP, matrix metalloproteinase; ND, not determined; VEGF, vascular endothelial growth factor.

the most common type of breast cancer is infiltrating ductal carcinoma. This class of tumors represents nearly three-quarters of all human breast cancers. Infiltrating lobular carcinomas account for 5-10% of breast carcinomas and are often characterized by multicentric tumors in the same or contralateral breast. Both ductal and lobular carcinomas have a predisposition for metastases to draining axillary lymph nodes, but each has differential predisposition for bone or visceral metastasis (Harris *et al.* 1984, Coleman *et al.* 1998). The molecular basis for these differences are mostly unknown. There are numerous other special types of invasive breast carcinomas. The most common are medullary, tubular and mucinous carcinomas. Medullary accounts for 5-7% of all breast carcinomas and are frequently well-circumscribed and exhibit lymphocytic infiltration (Fisher *et al.* 1990). Mucinous (or colloid) carcinomas account for 1-3% of breast carcinomas and are characterized, as their name implies, by accumulation of mucin around the tumor cells. Overall prognosis for mucinous tumors is better than ductal or lobular carcinomas.

Solely on the basis of their clinical behaviors, these are distinct types of breast carcinoma. It is likely that different genes are involved in controlling development and progression of each type. Yet most discussions of breast cancer genetics have not, for the most part, discriminated between each type of carcinoma. This is even more apparent when discussing the genetics of late-stage breast cancer. Since infiltrating ductal carcinomas are the most prevalent breast carcinoma type, most of the published results probably apply to ductal carcinomas, but this is not necessarily a good assumption (Larsson *et al.* 1990, Afify *et al.* 1996, Nishizaki *et al.* 1997, Toikkanen *et al.* 1997). There is, fortunately, a recent trend towards studying cancer genetics using more refined pathological criteria; however, more effort is required.

Further complications occur because of the use of cell lines which have been maintained in culture or passaged in animals for several years. The cells have probably undergone genotypic and phenotypic drift as well as selection pressures so that resemblance of the cell lines to the original tumor may be minimal. Sadly, although most breast carcinoma cell lines were derived from metastatic lesions, most no longer retain this ability in experimental systems (i.e. metastasis from mammary fat pads in immunocompromised (athymic or severe combined immunodeficiency (SCID) mice). This limitation severely hinders the ability of investigators to assess directly the metastasis-regulatory effects of individual genes. Given these caveats, any generalizations should be viewed with healthy skepticism. Nonetheless, certain patterns emerge and allow us to make a reasonable first approximation for a model of the molecular underpinnings of breast cancer progression and metastasis.

Oncogenesis and tumor progression are linked, but distinct, phenotypes

One area of confusion relates to terminology. Sloppy use of, and dual meanings of, some terms (depending upon one's specialization) are prevalent in the literature. Of particular relevance to this review are the distinctions between tumorigenesis vs tumor progression and malignant vs metastatic. Tumorigenesis and oncogenesis refer to the ability of cells to proliferate continuously in the absence of persistent stimulation by the triggering agent(s). Tumor progression is the evolution of already tumorigenic cells (populations) towards an increasingly autonomous state (i.e. decreased dependence upon host-derived growth factors and/or increased resistance to negative regulatory molecules). The distinction between oncogenesis and progression is crucial when asking whether a gene is important in controlling steps associated with malignancy, as compared with whether that gene is involved in tumor formation.

The distinctions between malignant and metastatic are more subtle. Attributes of malignant cells include (but are not limited to) less differentiated morphology, less differentiated cytology, level of vascularity, level of necrosis, mitotic index, aneuploidy, nuclear:cytoplasmic ratio. The incontrovertible hallmarks of malignancy are invasion of cells through a basement membrane and/or metastasis. All other characteristics used to label a tumor (and the cells within it) as malignant have exceptions (Pfeifer & Wick 1995). For example, morphologically indolent cells may be behaviorally malignant and vice versa. Clearly, parameters associated with pathological examination are invaluable when estimating the probability for local, regional or distant recurrence in a clinical setting. Nonetheless, subjectivity leads to ambiguity when trying to assign responsibility for a phenotype (i.e. metastasis).

Metastasis is defined as the formation of secondary tumor foci discontinuous from the primary tumor. The metastases can be nearby or at distant sites. Metastases can form following dissemination of cells via the lymphatic system, hematogenous system, coelomic cavities or epithelial cavities. Since they are by far the most common routes for metastatic spread of human breast cancer, lymphatic and hematogenous metastasis will be the focus here. In order to metastasize, cells must complete every step of a complex cascade. Malignant cells invade adjacent tissues and penetrate into the lymphatic and/or circulatory systems. Then tumor cells detach from the primary tumor and disseminate. During transport, cells travel individually or as emboli composed of tumor cells (homotypic) or tumor cells and host cells (heterotypic). At a secondary site, cells or emboli arrest either because of physical limitations (e.g. too large to traverse a capillary

lumen) or by binding to specific molecules in particular organs or tissues. Once there, tumor cells then proliferate either in the vasculature or, after extravasation, into surrounding tissue (Chambers *et al.* 1995, Koop *et al.* 1996). To form macroscopic metastases, cells must then recruit a vascular supply (Weinstat-Saslow & Steeg 1994, Ellis & Fidler 1995, Folkman 1995, Kohn & Liotta 1995) and respond appropriately to the tissue's environmental milieu (Nicolson 1994, Radinsky 1995). Fewer than 0.1% of cells that enter the vasculature survive to form clinically detectable, macroscopic metastases (Fidler 1970, Tarin *et al.* 1984). At which step(s) of the metastatic cascade circulating tumor cells commonly succumb is debatable (Chambers *et al.* 1995, Koop *et al.* 1995, 1996).

In the context of a multistep, multigenic cascade, it is critical to recognize that the terms invasiveness and adhesion are not equivalent to metastatic propensity. Both invasion and adhesion are necessary, but not sufficient for metastasis. Cells that are efficient at either or both — but which lack the ability to complete any other step of the metastatic cascade — are non-metastatic (Fidler & Radinsky 1990). Therefore, correlations of genetic expression to a particular step in the metastatic cascade may lead to erroneous conclusions.

Taken together, these points emphasize the importance of distinguishing tumor-suppressor and metastasis-suppressor genes. The former predominantly inhibit tumor formation when wild-type expression is restored in a neoplastic cell. By definition, then, metastasis would also be suppressed (since the cells are non-tumorigenic). Metastasis-suppressor genes, on the other hand, block only the ability to form metastases. Restoring expression of a metastasis-suppressor would yield cells which are still tumorigenic, but are no longer metastatic.

At diagnosis, breast carcinomas are typically mixtures of genotypically and phenotypically distinct cells, despite having arisen from a single cell (Welch & Tomasovic 1985, Fujii *et al.* 1996*a*,*b*, Rebbeck *et al.* 1996, Shows *et al.* 1997). One of the earliest detectable changes in transformed cells (anchorage-independent, not contact-inhibited, immortal but not necessarily able to form a tumor in an appropriate host) is a several-fold increase of genomic instability compared with normal cells (Ling *et al.* 1985, Cheng & Loeb 1993, Tlsty *et al.* 1993, Tlsty 1997). Karyotypic and genomic instability is present in transformed cells even before they acquire tumorigenic potential (Otto *et al.* 1989, Tlsty 1990, 1993, Jonczyk *et al.* 1993). Thus, genomic instability appears to be the driving force by which cells acquire the cumulative genetic defects necessary to be fully tumorigenic. Likewise, the development of heterogeneity, coupled with selective pressures results in continued evolution of the tumor population, usually towards increasing autonomy from the host (Foulds 1954, Heppner 1984, Welch & Tomasovic 1985, Heppner & Miller 1997). Eventually, some subpopulations of cells within the mass are amply self-sufficient that they have the ability to metastasize. This does not imply that metastatic cells do not respond to host-derived growth signals. Rather, it means that they do not necessarily require them. In conclusion, oncogenesis is a prerequisite for metastasis formation. In other words, metastatic cells represent a subset of tumorigenic cells.

One measure of genetic instability is microsatellite instability. Several reports have suggested that microsatellite instability is a useful prognostic indicator for breast cancer (Patel *et al.* 1994, Yee *et al.* 1994, Paulson *et al.* 1996); however, a role in development of metastasis has not been established. Recently, another means for developing genetic instability in non-hereditary nonpolyposis coli colorectal cancers was described (Cahill *et al.* 1998). Defective segregation machinery results in unequal partitioning of chromosomes in daughter cells, leading to aneuploidy. While it is common for breast carcinomas to be aneuploid, it has not yet been determined whether a similar mechanism is taking place in breast. Regardless of mechanism, genetic instability has practical consequences with regard to our ability to isolate and characterize metastasis-associated genes — key genetic changes are sometimes clouded by background 'noise' due to heterogeneity. Techniques such as tissue microdissection are now being utilized to minimize this problem (Zhuang *et al.* 1995).

Therefore, the ability to establish a role for a given gene in breast cancer metastasis is complicated by a variety of factors. The following discussion will focus on those genes for which genetic manipulation has been utilized to establish a role in controlling metastasis. Largely, the results are based upon experimental systems. Combined with clinical correlations, there is substantial evidence for controlling the metastatic potential of breast carcinoma.

The use of knockout and transgenic mice to study various aspects of breast cancer biology has been increasing in recent years (reviewed in (Thomas & Balkwill 1994, Amundadottir *et al.* 1996, Clarke 1996, Bennett & Wiseman 1997, Li *et al.* 1998). The use of such models has focused on tumor development rather than the later stages of tumor progression and metastasis. While improvements are occurring at a rapid rate, the models are still limited by relatively poor mimicry of the pathogenesis of human breast cancer.

Metastasis-controlling genes in breast carcinoma

Since a working model for tumorigenesis involves mutations of key genes that control cell growth and/or death, it appears plausible that metastasis will also be

controlled by a select set of genes controlling key steps in the cascade. On the basis of this presumption, we will focus on genes that appear likely to be important in either the suppression or promotion of breast cancer metastasis. In this regard, the genetic basis of metastasis would parallel the genetics of tumor formation. Evidence shows that metastasis involves numerous genes (Fidler & Radinsky 1990, Chambers & Matrisian 1997, Price *et al.* 1997, Welch & Goldberg 1997) that fall into two categories — (1) genes that drive metastasis formation, and (2) genes that inhibit metastasis (Dear & Kefford 1990, Welch *et al.* 1994, De La Rosa *et al.* 1995, Dong *et al.* 1995, Lee *et al.* 1996, Phillips *et al.* 1996, Lee & Welch 1997*b*). The number of identified metastasis-associated genes are growing rapidly. However, their mechanisms of action, their regulation in normal and/or cancer cells, and the universality of function in cancers of different origin remain largely unknown.

The best characterized dominantly acting metastasis gene (i.e. met-oncogene, which drives conversion from benign to malignant) is the activated ras oncogene (Collard *et al.* 1987, Chambers *et al.* 1990, Phillips *et al.* 1990). Transfection and constitutive expression of non-senescent rodent fibroblasts with activated Ha-ras leads to development of tumorigenic and metastatic properties (Muschel *et al.* 1985, Egan *et al.* 1987). However, complete induction of metastasis does not occur in all cell lines or cell types (Chambers *et al.* 1990, Tuck *et al.* 1990, Jessell & Melton 1992), nor is retention of ras oncogene expression necessary to maintain the metastatic phenotype (Schlatter & Waghorne 1992). In human breast cancer, overexpression of normal or mutant ras in human breast cancer has been associated with increased malignant properties (e.g. reduced responsiveness to estrogens, increased invasiveness, morphological abnormalities (Lundy *et al.* 1986, Theillet *et al.* 1986, Fromowitz *et al.* 1987)), but association with metastatic potential has not been unequivocally demonstrated. Mutations of ras, *per se*, are relatively uncommon in human breast cancer; so, the importance of ras in controlling breast cancer metastasis is not completely understood.

The prototypical metastasis-suppressor gene, Nm23, was first identified in the murine K1735 melanoma using subtractive hybridization, and its expression is inversely correlated with lung colonization (Steeg *et al.* 1988, Bevilacqua *et al.* 1989); however, there are exceptions (Radinsky *et al.* 1992). The human homolog, Nm23-H1 (also known as NME1), exhibits decreased expression in late-stage, metastatic human breast, endometrial, ovarian, melanoma and colon cancers (reviewed in Freije *et al.* 1996). However, long-term prognostic value has been questioned in some studies (Kapranos *et al.* 1996, Russell *et al.* 1997). Nonetheless, NME1 is a bona fide metastasis-suppressor gene in human breast carcinoma, since transfection of metastatic MDA-MB-435 cells resulted in a significant suppression of metastasis from the mammary gland in experimental mouse models (Leone *et al.* 1993). The mechanism of action for NME1 remains unknown (De La Rosa *et al.* 1995), but motility of the transfectants was significantly suppressed (Kantor *et al.* 1993). NME1 is homologous to *Drosophila* awd and encodes a 17 kDa protein. NME1's nucleoside diphosphate kinase homology (Biggs *et al.* 1990) and function (Steeg *et al.* 1991) have recently been dissociated from its metastasis-suppressor function (MacDonald *et al.* 1993, Royds *et al.* 1994, De La Rosa *et al.* 1995). Some recent reports suggest that NME1 may be involved in controlling cell cycle progression (Cipollini *et al.* 1997) and histidine-dependent protein phosphorylation reactions (Freije *et al.* 1997).

The story for Nm23 becomes more complicated because three additional family members (Nm23-H2/NME2, Nm23-DR, Nm23-H4) have recently been identified and cloned. NME2 has been shown to regulate transcription of the (proto)oncogene, c-myc (Postel *et al.* 1993, Berberich & Postel 1995, Ji *et al.* 1995, Seifert *et al.* 1995). Some studies have shown that NME2 can suppress metastasis (Engel *et al.* 1993, Mandai *et al.* 1994, Marone *et al.* 1996), whereas others have not (Arai *et al.* 1993, Tokunaga *et al.* 1993, Yamaguchi *et al.* 1994, Baba *et al.* 1995). Nm23-DR is differentially expressed during myeloid differentiation (Venturelli *et al.* 1995) but association with metastatic potential has not yet been tested in either clinical samples or experimental systems. Nm23-H4 differs structurally from the other homologs in that it appears to have additional N-terminal basic amino acid residues (Milon *et al.* 1997). However, its mechanism of action and relevance to breast cancer biology have not yet been reported.

A recent study even suggests that expression levels of Nm23-H1 in human breast cancer cell lines (HT115 and MDA-MB-231) can be influenced by diet. Increased consumption of linoleic and arachidonic acids reduced expression, whereas linolenic acid increased expression (Jiang *et al.* 1998). These conditions lowered invasiveness as measured by *in vitro* invasion assays. While a significant amount of work needs to be done to determine whether dietary regulation of metastasis is mediated through modulation of Nm23, dietary fat intake has been shown to control breast and mammary tumor metastasis (Hubbard & Erickson 1987, Rose *et al.* 1994, 1995).

KAI1 (also known as CD82 or C33, members of the TM4SF superfamily of adhesion molecules) was recently discovered as a prostate cancer metastasis-suppressor gene on the p-arm of chromosome 11 (Dong *et al.* 1995). Other members of the TM4SF family, namely MRP-1/CD9 and CD63/ME491, have been associated with metastatic potential of non small-cell human lung

carcinomas (Ikeyama *et al.* 1993) and early stage melanomas (Hotta *et al.* 1988) respectively. Thus, a role for KAI1 in breast cancer metastasis was possible. To test this hypothesis, we measured KAI1 mRNA expression in a panel of human cell lines representing a continuum from nearly normal breast cells (MCF10A) to highly metastatic cells (MDA-MB-435). KAI1 mRNA expression decreased with increasing invasive and metastatic potentials (Yang *et al.* 1997).

Lower KAI1 expression in metastatic breast cancers correlated well with previous findings that chromosome 11 deletions are common in late-stage breast carcinoma (Devilee & Cornelisse 1990, 1994, Mars & Saunders 1990, Negrini *et al.* 1995, Trent *et al.* 1995). To test directly whether changes on chromosome 11 were responsible for suppressing metastatic potential, we introduced a normal chromosome 11 into metastatic MDA-MB-435 breast carcinoma by microcell-mediated chromosomal transfer. Chromosome 11 significantly reduced the metastatic properties without affecting tumorigenicity (Phillips *et al.* 1996). Since KAI1 expression was higher in the chromosome 11 hybrids, we hypothesized that KAI1 is the gene responsible for suppressing metastasis. Expression of another TM4SF family member, TAPA-1 which is also encoded on chromosome 11, did not correlate with metastatic potential. Transfection and stable constitutive expression of KAI1 in MDA-MB-435 cells suppressed metastasis from tumors following injection into the orthotopic site — mammary fat pad (Phillips *et al.* 1998). However, the cell lines did not maintain transgene expression levels following *in vivo* growth. This complicated interpretation. Preliminary studies using a panel of human breast specimens of varying grades indicate that KAI1 protein staining was inversely related to grade of disease (XH Yang, LL Wei, C Tang & ME Lippman, unpublished observations). Nonetheless, KAI1 appears to meet the criteria described above for a metastasis-suppressor gene in human breast cancer.

Chromosome 1q deletions occur with variable frequency in late-stage human breast carcinomas. Since the recently discovered melanoma metastasis-suppressor gene, KiSS-1, maps to chromosome 1q32 (Lee *et al.* 1996), we tested whether KiSS-1 could suppress metastasis of the human breast ductal carcinoma cell line, MDA-MB-435. Parental MDA-MB-435 cells did not express KiSS-1, but non-metastatic MDA-MB-231 breast carcinoma cells did. Transfection of a full-length, constitutive mammalian expression construct suppressed metastasis of MDA-MB-435 from the mammary fat pad of athymic mice, whereas vector-only transfectants were unaffected (Lee & Welch 1997*b*).

The mechanism of action for KiSS-1 has not yet been determined, although its ability to suppress metastasis has been demonstrated in six independently-derived human cancer cell lines of melanoma and breast origin (Lee *et al.* 1997, Lee & Welch 1997*a*,*b*). Based upon the cDNA sequence, the predicted KiSS-1 protein would be a hydrophilic, 164 amino acid protein with molecular mass of 15.4 kDa. The sequence is novel, having no strong homology to any known human cDNA sequences. Four regions within the predicted KiSS-1 protein match consensus as phosphorylation sites for protein kinase C, protein kinase A and a tyrosine kinase (Lee *et al.* 1997). These sequences suggest that KiSS-1 is a phosphoprotein and our working hypothesis is that it functions within a signal transduction pathway. Thus far, KiSS-1 expression has never been detected in any cells that have metastatic potential. However, all studies have measured mRNA expression since antibodies are not yet available. This deficiency limits our ability to measure clinical correlations, although this is certainly a high priority goal.

Other metastasis-promoting or invasion-promoting genes have been identified in a variety of human and rodent tumor models. The genes include TIAM-1 (Habets *et al.* 1994), mts1 (Grigorian *et al.* 1994), mta1 (Toh *et al.* 1994), TI-241 (Ishiguro *et al.* 1996), fibroblast growth factor-4 (Dickson & Lippman 1992, McLeskey *et al.* 1996), and cathepsin D (Rochefort *et al.* 1990*a*,*b*). Transfection of these genes into experimental cell systems (usually fibroblasts) is reported to increase invasiveness and metastasis. Again, the definitive roles of these genes in mammary or breast cancers are not well-defined.

Protein kinase C (PKC) activities are important for several physiological processes relevant to mammary tumor promotion and progression (e.g. proliferation, motility, anchorage-independent growth, responses to growth factors, etc.). In collaboration with Drs Susan Jaken, Sue Kiley and Daniel Medina, we recently compared PKC isoenzyme levels in mouse and rat mammary tumor cell lines (S C Kiley, K Clark, S K Duddy, D R Welch & S Jaken, unpublished observations; Kiley *et al.* 1996, Jaken *et al.* 1997). Of particular relevance to this review, 13762NF mammary adenocarcinoma cell clones that have low, moderate and high metastatic potentials were evaluated for expression of PKCs α, δ, ε and ζ. All isoforms were expressed in each of the cell lines; however, PKCδ was significantly greater in highly metastatic compared with poorly metastatic cells. To determine whether this correlation was physiologically relevant, transfections were carried out to increase (full-length PKCδ cDNA in constitutive and inducible expression constructs) or decrease (dominant negative PKCδ regulatory domain (RDδ) in inducible expression constructs) PKCδ expression. Increased expression of PKCδ enhanced clonogenicity in soft agar and metastatic potential, but did not affect anchorage-dependent growth. Expression of the RDδ inhibited metastasis when cells

were injected into syngeneic rats. Moreover, induction of the RDδ with doxycycline (which induces the tetracycline-inducible promoter) caused a significant reduction in metastatic potential. Taken together, our results strongly imply that PKCδ is an important regulator of mammary tumor metastasis. Experiments are under way to determine the relevance of RDδ in controlling human breast cancer metastasis.

Chromosomal changes in breast cancer may predict the location of metastasis-controlling genes

As alluded to above, consistent, non-random re-arrangements, deletions and/or amplifications have been instrumental in identifying oncogenes and tumor-suppressor genes involved in the development of human cancer. Over 56 distinct regions of loss of heterozygosity (LOH) have been identified in breast cancer (Kerangueven *et al.* 1997). The frequency of involvement of each ranges from 20% to >50% depending upon the study, tumor type and markers used. Unfortunately, as tumors progress, they accumulate changes, leading to complex karyotypes. Structural or numerical aberrations for virtually every chromosome have been described in human breast cancer (see Table 2 for an example). Experience has told us that some of the chromosomal changes occur at a frequency higher than could be explained on a random mutational basis. These findings increase the probability that genes associated with tumor progression will be encoded at those sites. LOH has been found in chromosomal regions correlating with parameters associated with breast cancer progression/metastasis (see Table 3). To emphasize the point made above — i.e. that different types of breast cancer exhibit different chromosomal changes — Nishizaki and colleagues (1997) used the comparative genomic hybridization technique to compare lobular and ductal carcinomas. Lobular carcinomas had increased copies of DNA from chromosome 1q in 79% of patient samples and losses of chromosome 16q in 63%. The lobular carcinomas showed higher frequency of 16q loss than ductal carcinomas and lower frequency of 8q and 20q gains (Nishizaki *et al.* 1997).

In metastases vs primary tumors, karyotypic abnormalities of chromosomes 1, 6, 7, and 11 are particularly prevalent. Among the more common cytogenetic changes in metastases from breast is amplification in the region surrounding band q13 on chromosome 11. The amplicon includes the following genes: int-2 gene (which is syntenic to a site of frequent mouse mammary tumor virus (MMTV) insertional mutagenesis in mice (Lee *et al.* 1995), but the protein is not usually expressed in human breast tumors); hst (which is a member of fibroblast growth factor (FGF) family but this is not expressed at the mRNA level (Nguyen *et al.* 1988, Theilet *et al.* 1989)); bcl-1 (which was discovered by involvement in chromosomal translocations in some lymphomas (Tsujimoto *et al.* 1984, Theillet *et al.* 1990)); and PRAD-1 (which was initially discovered in parathyroid adenomas (Motokura *et al.* 1991, Motokura & Arnold 1993), but subsequently found to be cyclin D1 (Motokura *et al.* 1991, Motokura & Arnold 1993)). Amplification in this region is associated with poor prognosis (Lidereau *et al.* 1988, Tsuda *et al.* 1989), presence of lymph node metastases (Zhou *et al.* 1988, Theilet *et al.* 1989, Adnane *et al.* 1991), and ER and progesterone receptor (PR) status (Theilet *et al.* 1989, Fantl *et al.* 1990, Borg *et al.* 1991). While these correlations are compelling, definitive association of 11q13 amplification with metastatic potential has not been demonstrated.

As mentioned above, microcell-mediated chromosomal transfer of chromosome 11 reveals that there exists a metastasis suppressor activity on chromosome 11. However, these types of experiments are complicated because results vary according to the experimental models used. Microcell transfer into MCF7 breast cancer cells revealed that BrCa-1- and p53-independent growth inhibitors (i.e. inhibitors of tumorigenicity) are encoded on chromosome 17 (Casey *et al.* 1993, Theile *et al.* 1995, Plummer *et al.* 1997). Additional growth inhibitors have been described on chromosomes 6 and 11 (Negrini *et al.* 1994, Theile *et al.* 1996, Shows *et al.* 1997). Interestingly, transfer of chromosome 11 suppresses growth in culture and tumor formation in the MDA-MB-231 and MCF7 models, but neither phenotype was significantly, nor consistently affected in MDA-MB-435. These data clearly show that extrapolation based upon data from a single model is ill-advised. However, this problem is not easily solved because of a lack of relevant metastatic models of human breast cancer.

Inadequate models exist to study breast cancer metastasis

Despite the fact that the majority of human breast cancer cell lines have been derived from metastatic lesions, only MDA-MB-435 reproducibly forms macrometastases when evaluated in athymic or SCID mice (Price *et al.* 1990, Price 1996). This is a serious limitation for investigators wishing to study metastasis of human breast cancer. Several investigators have found that MDA-MB-231 will form lung metastases following injection into the mammary fat pad (Price *et al.* 1990, Rose *et al.* 1994) or bone metastases following intracardiac injection (Mbalaviele *et al.* 1996, Guise 1997). Interestingly, none

Table 2 Percentage of breast carcinomas showing chromosomal aberrations

	Chromosome																						
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**X**
Primary tumor																							
Structural (p-arm)	7	-	4	1	1	4	1	2	1	-	4	-	-	-	-	-	-	-	-	-	-	-	-
Structural (q-arm)	2	5	1	2	1	2	4	2	2	1	2	2	1	-	-	2	1	-	1	-	-	-	1
Numerical (gain)	-	-	-	-	1	-	2	-	1	-	1	-	-	-	-	-	-	-	-	1	1	-	-
Numerical (loss)	2	-	-	4	1	2	2	5	5	5	5	2	2	2	2	5	6	2	5	-	2	2	2
Metastases																							
Structural (p-arm)	22	-	18	6	10	8	8	8	10	-	8	10	4	-	2	-	2	-	-	-	-	-	2
Structural (q-arm)	20	12	10	4	4	12	16	4	6	2	14	10	4	2	2	6	2	-	-	-	4	2	-
Numerical (gain)	-	4	6	8	10	2	15	6	4	4	4	6	4	8	4	2	4	10	4	8	10	6	8
Numerical (loss)	10	14	6	6	8	6	4	4	6	8	10	10	8	8	6	8	6	6	6	4	6	10	4

Data presented here are adapted from Emerson *et al.* (1993); Hill *et al.* (1987); Trent *et al.* (1993) using karyotypic analyses of short term cultures from recently removed breast tissue (primary tumor or metastases). While the overall values vary by study, the relative involvement is consistent with other studies using comparative genomic hybridization (Devilee & Cornelisse 1994; Devilee *et al.* 1994; Gray *et al.* 1994; Kallioniemi *et al.* 1994).

of the models currently available metastasize to bone following tumor growth in the mammary fat pad, despite this being the most common site for metastasis in clinical breast cancer (Coleman 1997). Three points deserve emphasis. First, lung colonization efficiency is generally lower in MDA-MB-231 than in MDA-MB-435. If metastasis suppression is the desired biological endpoint, it is important that baseline levels be as high as possible. Secondly, as with MCF7 cells, there are several different sublines of MDA-MB-435 and MDA-MB-231 that have been artificially selected over the years in many different laboratories. Some of these cells are no longer tumorigenic in immunocompromised mice. Therefore, it is incumbent upon each investigator to verify metastatic potential in his/her laboratory. Thirdly, the distribution of metastatic lesions in immunocompromised mice does not completely mimic the clinical situation. While not inappropriate, the models are somewhat lacking in this regard.

Breast cancer metastasis is not solely due to genetic changes

A heritable component of the metastatic phenotype has been demonstrated numerous times by experimental isolation of metastatic and non-metastatic clones as well as selection of increasingly metastatic variants from heterogeneous tumor populations. For cells to metastasize successfully, they must also interact with a variety of host cells and their secreted molecules and respond appropriately. Thus, any discussion of factors controlling metastasis must include an evaluation of exogenous regulators of the process (or its component steps). Normal breast tissue growth, differentiation and regression after lactation are all exquisitely controlled by hormones. Indeed initiation, promotion and progression of breast carcinomas are strongly regulated by endocrine mechanisms (Dickson *et al.* 1993, Kaufmann 1997).

Hormones contribute to breast cancer development and metastasis

Hormones have long been implicated in the initiation, development, and progression of breast cancer. Numerous epidemiological studies spanning almost two decades have established that, excluding a genetic predisposition, the reproductive history of a woman is an important risk factor associated with the development of breast cancer. Early menarche and late menopause have been shown to be associated with an increased risk of breast cancer. Epidemiological studies also show that early pregnancy provides a protective effect against breast cancer, but that the protection declines as the age of first pregnancy increases. Taken together, these studies suggest that the length of time between menarche and menopause or menarche and first pregnancy are contributing factors towards the risk or likelihood of breast cancer oncogenesis (Staszewski 1971, Key & Pike 1988, Henderson *et al.* 1991).

Table 3 Chromosomal location of LOH and the correlation with parameters associated with breast cancer progression/metastasis

Chromosome region	Parameter	Reference
1p	Nodal status	(Borg *et al.* 1992*b*)
1p36,1p34-p35	Nodal status	(Tsukamoto *et al.* 1998)
1q21-q24	Stage	(Devilee *et al.* 1991)
3p21.3	Metastasis	(Driouch *et al.* 1998)
3p21-p25	LOH on 11p, 17p, 17q and aneuploidy	(Devilee *et al.* 1994)
7q23	Metastasis-free overall survival	(Bieche *et al.* 1992)
8p21.3-p23	Low grade DCIS	(Anbazhagan *et al.* 1998)
9q	LOH on 1q, 17p, 18q	(Devilee *et al.* 1994)
11p15	ER$^-$ tumors, grade III tumors and distant metastasis	(Ali *et al.* 1987)
11p15	Lymph node status	(Takita *et al.* 1992)
11p15.5-15.4	Histologic grade	(Karnik *et al.* 1998)
13q12-q14	ER content	(Devilee *et al.* 1994)
13q12-q14	Ductal carcinoma tumor size	(Andersen *et al.* 1992)
13q12-q14	Aneuploidy and S-phase fraction >12%	(Borg *et al.* 1992b)
16q22.2-q23.2	Metastasis	(Driouch *et al.* 1998)
16q23.2-q24.2	Good prognosis	(Hansen *et al.* 1998)
16q24	ER content	(Devilee *et al.* 1994)
17q12-q24	c-erb-B2 amplification	(Sato *et al.* 1991)
17q12-q24	Age of onset	(Devilee *et al.* 1994)
17q12-q24	c-erb-B2 amplification/post-menopausal status	(Andersen *et al.* 1992)

ER$^-$, estrogen receptor negative tumors; DCIS, ductal carcinoma *in situ*.

The two principal hormones involved both in the onset of menarche and in menopause are the female sex steroids, estrogen (specifically 17β-estradiol) and progesterone. It is well established that estrogen promotes breast cancer by stimulating cell division. Although the main source of estrogen is the ovary in premenopausal women, estrogen can also be synthesized directly in adipose tissue and breast cancer cells via the enzyme aromatase (Yue *et al.* 1998). Aromatization is typically thought to be the predominant source of estrogens in post-menopausal women (Brodie & Santen 1994, Harvey 1997, Kaufmann 1997). More controversial is the role that estrogens or estrogen metabolites can have in causing or initiating breast cancer. Recent findings suggest that metabolites of 17β-estradiol may be among the culprits leading to DNA damage and subsequently for initiation of breast cancer (Fishman *et al.* 1995, Cavalieri *et al.* 1997, Lavigne *et al.* 1997, Zhu & Conney 1998). However, this interpretation is debatable and additional research will be required to establish this definitively. Nonetheless, there is little doubt that estrogens play a key role in promoting initiated human breast cancer to grow and to progress.

A role for progesterone in breast cancer development is less clear than for estrogen. At one time, it was generally accepted that progesterone was a natural antagonist of estrogen action — suggesting that it would inhibit or block growth-promoting effects of estradiol on breast cells (normal and tumor). This paradigm was based upon findings in the uterus in which progestins reduced or eliminated the risk of estrogen-induced endometrial cancer. Recently, the effect of progesterone (analogs) on normal breast epithelial cells has been re-examined. The mitotic index of normal breast epithelial cells parallels changes in hormone levels during the menstrual cycle. In cyclic women, serum estrogen levels are highest during the follicular phase, with a secondary resurgence in the secretory phase. The mitotic index of endometrial cells parallels serum estrogen levels. In contrast, breast epithelial mitoses are greatest during the secretory phase when serum progesterone levels are maximal (Masters *et al.* 1977, Meyer 1977, Going *et al.* 1988). The latter raises the possibility that progesterone may have growth-promoting effects on breast epithelial cells. This supposition is further supported by the following lines of evidence: (1) progestins are mitogenic for established breast cancer cell lines *in vitro* (Hissom & Moore 1987, Hissom *et al.* 1989, Manni *et al.* 1991); (2) progestins promote growth of established mammary tumors (Huggins & Yang 1962, Huggins 1965, Robinson & Jordan 1987); (3) progestins stimulate expression of mitogenic growth factors and/or their receptors (Dickson & Lippman 1988, Murphy *et al.* 1988, Lanari *et al.* 1989, Murphy & Dotzlaw 1989, Papa *et al.* 1991); and (4) anti-progestins induce apoptosis in experimental mammary tumor models (Michna *et al.* 1989, Schneider *et al.* 1989). Thus, progesterone exposure may be a contributing factor towards the development of breast cancer (Groshong *et al.* 1997).

Estrogen and progesterone exert their cellular effects through interactions with nuclear receptor proteins called the estrogen receptor (ER) and the progesterone receptor (PR) respectively. The recognition that these receptors are the primary mediators of estrogen and progesterone action and that their presence within a tumor specimen can help predict the responsiveness of human breast cancer to hormonal therapy is particularly useful. Today, the measurement of ER levels is standard practice and is a useful prognostic marker in determining which patients are most likely to respond to estrogen antagonist therapies such as the anti-estrogen, tamoxifen (also known as Nolvadex). Since PR is an estrogen-induced product, simultaneous detection of PR in the presence of ER from a single tumor is indicative of a functional estrogen receptor pathway and further improves the ability to predict the response to anti-estrogen therapy. Alternatively, the absence of ER and PR is associated with early recurrence and poor survival of the breast cancer patient.

The ER mentioned above refers to the alpha ER (ER-α). Recently, a second ER form has been cloned (ER-β) (Kuiper *et al.* 1996). ER-α and ER-β both bind 17β-estradiol in traditional binding assays. However, current data suggest that the amount of ER-β relative to ER-α in breast cancer cells is minor (Kuiper *et al.* 1996, Petersen *et al.* 1998). In the normal mammary glands of mice, ER-β is undetectable (Couse *et al.* 1997). Whether ER-β will play an important role in breast cancer biology or etiology remains to be determined, although there have been reports of ER-β mutants in breast cancer cells (Dotzlaw *et al.* 1997, Vladusic *et al.* 1998). It is important to remember that many of the studies with ER-β are based upon mRNA, rather than protein, expression. Once more robust protein detection methods/reagents have been developed, the relative importance of ER-β in breast cancers, if any, will be more easily evaluated.

Since almost all breast cancers progress from a hormone-responsive state to a hormone-resistant or hormone non-responsive state, the possibility was raised that mutations in the ER-α (the predominant form of ER in breast cancers) could be a factor leading to anti-estrogen resistance in breast cancer. Several investigators pursued this line of thought and have shown that mutant ERs exist in some breast cancer cell lines and tumor specimens (Graham *et al.* 1990, Fuqua *et al.* 1991*a*, 1992, Scott *et al.* 1991, Wang & Miksicek 1991). Moreover, mutations of ER can lead to variant estrogen receptor activity which, in turn, may explain estrogen resistance (Fuqua *et al.* 1991*a*, 1992). Furthermore, from these and other studies that have

focused on ligand-receptor interactions, it is apparent that variations in ER structure and ligand-specific (estrogen versus anti-estrogen) interactions with ER may lead to altered and unexpected biological responses (Katzenellenbogen 1996, McInerney & Katzenellenbogen 1996, Montano *et al.* 1996, Levenson *et al.* 1997). This is further complicated by promoter and cell-specific factors (Katzenellenbogen 1996, Yang *et al.* 1996). Although the existence of mutant ERs is very appealing, their actual contribution to disease progression, particularly anti-estrogen resistance, appears to be small. Furthermore, most of the variant ER data to date have been found at the mRNA level. It is still not known whether they are translated into proteins (Dowsett *et al.* 1997, Murphy *et al.* 1997*a*,*b*, Tonetti & Jordan 1997).

Although less research has been dedicated towards the identification of variant PR, there are several papers reporting the existence of variant PR mRNA and protein (Wei *et al.* 1990, Wei & Miner 1994, Leygue *et al.* 1996*a*, Richer *et al.* 1998, Yeates *et al.* 1998). One variant PR protein form is N-terminally truncated compared with the previously reported A and B PR isoforms. This third form, the C-receptor, has unique transcriptional enhancing properties when in the presence of the two larger PR isoforms and ligand (Wei *et al.* 1996). From this work and the abundance of other studies, it is becoming apparent that steroid-regulated growth and gene expression involves multiple regulatory factors, of which the steroid receptor is but one component, and that the eventual biological outcome is dependent upon the interaction of steroid receptors with non-receptor proteins (i.e. adaptors) (Katzenellenbogen *et al.* 1996, Glass *et al.* 1997, Shibata *et al.* 1997). Several proteins to date have been associated with gene transcriptional enhancing properties such as SRC-1 (Onate *et al.* 1995, Spencer *et al.* 1997), AIB-1 (a member of the SRC-1 family) (Anzick *et al.* 1997) and RIP140 (Cavailles *et al.* 1995). Likewise, transcriptional repressor proteins have been identified (Chen & Evans 1995). Steroid-regulated gene expression is further complicated because some neurotransmitters and growth factors (e.g. epidermal growth factor) can activate steroid hormone receptors independently of ligand (Ignar-Trowbridge *et al.* 1992, Gangolli *et al.* 1997). Also, some steroid hormones can mimic growth factor action in the absence of steroid hormone receptors e.g. progesterone binds to oxytocin receptor (Grazzini *et al.* 1998) and estrogen receptor binds to cerbB-2 receptor (Matsuda *et al.* 1993). Collectively, these studies indicate that steroid-driven gene activation is modulated by multiple factors, of which only one component is the receptor. So, although estrogen and progesterone are key hormones in the regulation of breast cancer growth, there are many additional contributory factors (i.e. growth factors and co-factors) that also regulate breast cancer proliferation.

Although steroid hormone receptor levels can be used as a marker to assess the extent of tumor progression towards malignancy, few studies directly demonstrate a functional role in this regard, especially with regard to metastasis. The most direct test was by Garcia *et al.* (1992) who transfected the ER-negative MDA-MB-231 breast carcinoma cell line with estrogen receptor (ER-α) and then treated the transfectant cells with estrogens and anti-estrogens. Experimental metastatic potential following intravenous inoculation of cells was inhibited threefold by estradiol whereas the anti-estrogen tamoxifen had little effect (Garcia *et al.* 1992). Estradiol also increased the invasive capabilities of these transfectants in an *in vitro* invasion assay using Matrigel; anti-estrogens inhibited these effects. Interestingly, in contrast to the typical stimulatory effect of estradiol on ER-positive breast cancer cell growth, estradiol inhibited the cell proliferation of ER-transfectants. These results must be viewed cautiously until further experiments are done to explain this phenomenon or the experiments are replicated in another cell line.

Endocrine regulation does not act independently to regulate breast tumor cell behavior. The biochemical changes resulting from modified ligand and receptor expression and activation, combined with inter-relationships with other growth factors and intracellular signaling pathways, reveal a byzantine regulatory machinery. Abnormal tissue growth is due to a disruption of the balance between stimulated proliferation and inhibition of cell death. Transformation and progression can be due to: (1) increased production of growth-promoting factors; (2) decreased synthesis of growth-inhibitory factors; (3) decreased responsiveness to growth-promoting factors; or (4) decreased sensitivity to growth inhibitory signals. The latter two mechanisms can be direct, because of alterations in receptors, or via modifications in the downstream signaling pathways. For purposes of this review, only selected growth factors will be presented to provide examples of the complexities of growth regulation of breast cancer growth and progression.

Transforming growth factors

Transforming growth factors (TGFs) were identified initially and named based on their ability to transform selected cell types. This family of growth factors has expanded extensively and is now known to consist of several families of polypeptides (Hartsough & Mulder 1997). These are produced and secreted by normal and cancerous cells. TGF expression can be regulated by steroids as well as by other growth promoting factors, thereby leading to an intricate complex of negative and positive pathways modulating cell cycle progression or

homeostasis. TGF-α and TGF-β represent two distinct families of growth factors that are structurally and functionally distinct.

TGF-α and epidermal growth factor families

Many members of the TGF-α family compete with epidermal growth factor (EGF) for binding to the EGF receptor. Like EGF, TGF-α binding results in receptor dimerization, activation of tyrosine kinase activity and eventually leads to stimulation of cell proliferation or differentiation (Massague 1983, Derynck 1988, Todaro *et al.* 1990). Other members of this family include amphiregulin, heparin-binding EGF, cripto-1, and a subfamily of heparin binding proteins called heregulins (the human homolog) (Bates *et al.* 1988, Todaro *et al.* 1990, Higashiyama *et al.* 1991). Heregulin does not appear to bind the classic EGF receptor, but initially was thought to bind instead to a related EGF receptor protein called erbB-2 (HER-2/neu) (Schechter *et al.* 1984, 1985, Coussens *et al.* 1985, Bargmann *et al.* 1986, Stern *et al.* 1986, Yamamoto *et al.* 1986). Studies now indicate that heregulin does not bind directly to erbB-2, but rather to two related receptor forms, erbB-3 (Kraus *et al.* 1989, Plowman *et al.* 1990) and erbB-4 (Plowman *et al.* 1993, Carraway *et al.* 1994). All four receptor forms (EGF receptor, erbB-2, -3 and -4) have been reported to be present in human breast cancers. In about 30% of human breast cancers, erbB-2 is amplified or overexpressed; this is associated with poor patient prognosis and maintenance of the malignant phenotype (Slamon *et al.* 1987, Allred *et al.* 1992).

Overexpression of erbB-2/HER-2/neu and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancer (Van de Vijver *et al.* 1988, De Potter *et al.* 1990, Paik *et al.* 1990, Allred *et al.* 1992, Gusterson *et al.* 1992, Toikkanen *et al.* 1992). Because of this, erbB-2 overexpression was thought to be a key factor that increased the invasive potential of breast cancer cells; however, studies examining comedo-type intraductal carcinomas showed that a higher proportion overexpressed erbB-2 protein compared with invasive cancer, thereby indicating that, although erbB-2 overexpression may play a role in invasion, it does not singly lead to increased invasiveness (Van de Vijver *et al.* 1988). The roles of erbB-3 and -4 in breast cancer invasion and metastasis are not known.

TGF-β family

The TGF-β family of polypeptide growth factors is comprised of several related gene products that form either homodimers or heterodimers. TGF-β isoforms are found in both normal mammary epithelium and in breast tumors. The interactions of these various isoforms is further complicated by the presence of specific binding proteins (Chefietz *et al.* 1988, Murphy-Ullrich *et al.* 1992, Wakefield *et al.* 1992, Butzow *et al.* 1993). In addition, two TGF-β receptors (type I and type II) have been identified. Four type I receptors have been cloned (Wang *et al.* 1994*b*). Type I and type II receptors can heterodimerize. Because there are a wide variety of receptor combinations as well as the existence of multiple TGF-β forms, a diverse number of pathways appear available to regulate breast cancer growth and differentiation.

Most normal epithelial cells are growth inhibited when exposed to TGF-β (Arteaga *et al.* 1996). Restoration of TGF-β receptors in non-responsive MCF7 cells renders the cells less tumorigenic and less proliferative when grown in the presence of TGF-β (Sun *et al.* 1994). Because of this, studies on the role of TGF-β in cancer biology have mostly focused on this factor's effect on growth regulation and tumor formation. However, there is accumulating evidence that TGF-β plays a critical role in tumor invasion and metastasis.

TGF-β overexpression in breast tumors has been associated with a more malignant phenotype (Dickson & Lippman 1996). A specific role in invasion and metastasis was demonstrated when Welch and colleagues (1990) first showed that exposure of mammary adenocarcinoma cell lines to picomolar concentrations of TGF-β1 or TGF-β2 induced production of metalloproteinases, with a corresponding increase in invasiveness and experimental metastatic potential. At these concentrations, growth inhibition was not observed. Similar findings have been reported for the metalloproteinases as well as the urokinases (Walker & Dearing 1992, Agarwal *et al.* 1994, Walker *et al.* 1994, Sehgal *et al.* 1996, Reiss & Barcellos-Hoff 1997, Dong-Le *et al.* 1998). It is important to note that the source of the TGF-β can be the tumor cells themselves or nearby host cells. Indeed TGF-β can increase stromal cell secretion of urokinase (Hildenbrand *et al.* 1998). Thus, tumor cells which produce TGF-β could manipulate stromal cells to assist in their malignancy. This concept is substantiated by the known roles of TGF-β in angiogenesis and immunosuppression (Enenstein *et al.* 1992, Relf *et al.* 1997, De Jong *et al.* 1998*a*,*b*).

Interestingly, TGF-β expression was originally correlated with increased bone colonization by Walker 256 carcinosarcoma cells (Orr *et al.* 1993). Since bone is the most common site for breast cancer metastasis, organotropism may be partly explained by differential expression of TGF-β. This hypothesis is, at least partially, supported by Guise and colleagues who showed that TGF-β can alter expression of parathyroid hormone-

related protein (PTHrP) which is, in turn, involved in bone resorption. Expression of PTHrP, with or without exposure to TGF-β, regulates bone colonization by MDA-MB-231 cells (Guise 1997). Still, it must be emphasized that a role for TGF-β in bone colonization by breast cancer has still not been definitively established.

Other growth factors

In addition to the EGF and TGF-β families, numerous other growth factor families have been identified and found in breast cancer cells. These include the insulin-like growth factors (IGF-I and IGF-II), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and vascular endothelial growth factor (VEGF) (Heldin & Westermark 1984, Goustin *et al.* 1986, Sporn & Roberts 1986, Ferrara *et al.* 1992). The expression of many of these growth factors can be regulated by estrogen and progesterone (Dickson & Lippman 1996).

Thrombospondin is a 450 kDa adhesive glycoprotein present in high concentrations in the platelet alpha-granule. It is also synthesized by other cells and is incorporated into extracellular matrices. The role of thrombospondin in breast cancer biology is checkered (Walz 1992, Volpert *et al.* 1995, Qian & Tuszynski 1996, Roberts 1996). Transfection experiments suggest that it can promote cell adhesion, invasion and/or metastasis in some tumor models (Tuszynski *et al.* 1987*a*, Pratt *et al.* 1989, Walz 1992, Arnoletti *et al.* 1995, Incardona *et al.* 1995, Wang *et al.* 1996), whereas it is suppressive in others (Weinstat-Saslow *et al.* 1994, Zabrenetzky *et al.* 1994, Qian & Tuszynski 1996). Metastasis-promoting effects are often attributed to changes in adhesion whereas the suppressive effects can be, at least partially, explained by the anti-angiogenic effect of thrombospondin (Dameron *et al.* 1994*a*,*b*, Weinstat-Saslow *et al.* 1994, Volpert *et al.* 1995). Interestingly, thrombospondin expression is regulated by progesterone in the endometrium (Iruela-Arispe *et al.* 1996), opening the possibility that analogous regulation could occur in the breast. Also, thrombospondin-1 (TSP-1) expression appears to be regulated by p53 (Dameron *et al.* 1994*b*), which itself has been implicated in breast tumorigenesis (see TP53 in Table 1).

Thus, there are a multitude of interrelated growth factors, receptor types, and steroid hormones in the normal mammary epithelium that tightly regulate and coordinate cell proliferation and differentiation. In breast cancer cells, the intricate balance is perturbed. Invasive and metastatic cells further circumvent the regulation by overexpression or downregulation of growth factors and/or their receptors. Aberrations of downstream signaling cascades further contribute to cellular delinquency. Delineation of these pathways and their impact on angiogenesis, immune response, growth, invasion, and metastasis will require new models.

Immune regulation of breast cancer metastasis

There is clear evidence that breast cancer metastasis is based upon the inherent genetic makeup of the tumor cells. However, tumor cells do not exist in isolation and their biological properties are not fully self-determined. Examples are described above but there is one more that merits mentioning. The role of the immune system in cancer is usually considered to be the elimination of tumor cells; however, because metastatic cells and activated leukocytes share many properties, including the ability to attach to endothelium (Hoover & Ketcham 1975, Yong & Linch 1993) as well as degradation of and penetration of basement membranes (Wright & Gallin 1979, Klotz & Jesaitis 1994), it was suggested that, under certain conditions, tumor cells might exploit normal leukocyte function to increase metastatic efficiency (Gorelik *et al.* 1982, Aeed *et al.* 1988).

Rats injected with syngeneic 13762NF mammary adenocarcinoma cell clones developed neutrophilia (i.e. tumor-elicited neutrophilia) proportional to the metastatic potential of the primary tumor (Aeed *et al.* 1988). We showed that the metastatic tumor variants did so by secreting granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or interleukin-3 (IL-3) in proportion to their metastatic propensity (McGary *et al.* 1995). More importantly, tumor-elicited neutrophils increased metastatic potential and invasiveness of breast cells 2- to 25-fold when co-injected intravenously (Welch *et al.* 1989), whereas normal circulating neutrophils, proteose peptone-elicited and activated neutrophils and phorbol ester-activated neutrophils did not. Alone, these findings may have been merely an experimental curiosity. However, anecdotal clinical data suggest that these types of observations are not altogether uncommon. Leukocytosis (Sawyers *et al.* 1992), granulocytosis (Hughes & Higley 1952, Suda *et al.* 1980), eosinophilia (Sawyers *et al.* 1992) and neutrophilia (Lee *et al.* 1987) have been described in patients with advanced neoplasms of multiple histological types. This could not be explained solely by infection or tumor necrosis (Aeed *et al.* 1988). In experimental models, the evidence predominantly supports secretion of factors that stimulate bone marrow precursor cells. Lee and colleagues have shown that GM-CSF levels may be correlated with more advanced mammary tumors (Lee & Baylink 1983, Lee & Lottsfeldt 1984, Lee *et al.* 1987). Factor(s) produced by other tumor cell types that elicit bone marrow proliferation vary by tumor type, stage and size (Asano *et al.* 1977, Wu *et al.* 1979, Mano *et al.* 1987, Fu *et al.* 1991, Nitta *et al.* 1992,

Sawyers *et al.* 1992). Takeda *et al.* (1991) found that 7/14 metastatic transplantable tumors produced GM-CSF mRNA and/or detectable GM-CSF activity, whereas the non-metastatic tumors did not. Taken together, these results demonstrate that breast cancers may modulate their metastatic potential, in part, by manipulation of the immune system.

A molecular genetic model for breast tumor progression

The collection of neoplastic breast diseases are sufficiently distinct that it is unlikely that a single model could describe the genetic changes leading to metastasis. At the root of any model must be a clear understanding of the cell type from which a particular neoplasm developed. Notwithstanding, the majority of evidence suggests that cells from the terminal ductal structures are the cells of origin. Insufficient biochemical and molecular markers defining each breast cell type allow for more refinement than that with regard to cellular origin of breast neoplasms. It is believed that the conversion to neoplasia has an intermediary atypical hyperplasia in which the cells have lost some aspects of growth control, but still retain vestigial response to growth controlling signals. During the proliferative phase, cells are responding to the usual milieu of positive and negative endocrine, paracrine and juxtacrine signals. During this hyperproliferative phase, breast epithelial cells accumulate mutations in oncogenes and tumor suppressor genes so that they appear even less 'normal' or differentiated and are classified as carcinomas *in situ*. Further proliferation results in accumulation of mutations, increasing malignant characteristics (i.e. invasion, aneuploidy, angiogenesis, etc.), so that eventually a subset of cells is no longer confined to the breast.

Over 150 genes and genetic loci have been associated with breast cancer development. Of those changes, this review summarizes evidence implicating a role in progression to malignancy for over forty different genes. The magnitude of these numbers highlight the tremendous complexity of breast cancer as a family of diseases. The good news is that all of these markers have been identified, in spite of the extraordinary heterogeneity that exists within breast neoplasms at diagnosis. The bad news is that these changes are only the tip of the iceberg. How, then, can one determine which changes are essential and which are ancillary?

For oncogenes and tumor suppressor genes, the data in breast cancer oncogenesis are relatively mature. While there is still plenty of room for further study, correlative data are often corroborated by functional studies (i.e. transfection with wild-type cDNA followed by bioassay). The mechanism of action is not always known; however, the biological endpoints are unambiguous. The situation is less clear with regard to genes/loci involved in breast tumor progression, invasion and/or metastasis. Only four genes (Nm23-H1, KiSS-1, KAI1 and TSP-1) have been demonstrated to suppress metastasis of human breast carcinoma cells following orthotopic implantation of tumor cells into immunocompromised mice. Of these, only one, NME1 has been studied adequately in the clinical arena to warrant serious consideration as having prognostic value. KAI1 suppressed metastasis at a level comparable to Nm23, but KiSS-1 was more potent than any of the other genes tested with regard to reduction in metastasis incidence. To claim TSP-1 as a metastasis-suppressor gene may be a misnomer since tumor growth was also inhibited. Nonetheless, the tumor cells still expressed the transgene, allowing TSP-1 still to qualify by the criteria listed above.

Considering the number of papers claiming to study metastasis of breast cancer, the number of *bona fide* functionally tested metastasis-suppressor genes is surprisingly small. In part, this is due to the paucity of models which allow testing *in vivo*. Indeed most of the functional studies were done using the MDA-MB-435 model. Validation in other models has not been done. Certainly, testing in other breast tumor types has not been attempted. Thus, for the breast cancer metastasis field to advance further, more and better models will be required.

Despite the discovery and identification of four (and probably more) metastasis-suppressor genes, several questions remain regarding control of the metastatic phenotype in human breast cancer. Do the identified genes represent rate-limiting steps? Are these genes functioning in a single pathway or convergent pathways of metastasis control? What are the signals that control these genes? Are the key controlling signals among the correlations already established for breast cancer progression (i.e. hormonal or growth factor control)? While much has been learned, more still remains to be found.

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